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STAINING OF NORMAL, ATYPICAL, AND CANCEROUS COLON EPITHELIUM BY OVALBUMIN-FLUORESCEINISO-THIOCYANATE AT DIFFERENT pH LEVELS

By

JAN HULTÉN and JAN PONTÉN

Received 24 v 63

During the last few years, *P E Hughes, C J Louis* and collaborators have developed a staining process which makes possible specific differentiation between neoplastic and non neoplastic epithelium (3,7,8) (For further references, see 9) Frozen sections were stained by proteins conjugated to fluoresceinisocyanate With proper technique, neoplastic and preneoplastic cells were left unstained, whereas normal, embryonic or regenerating ephithelium stained brilliantly It was suggested that the conjugation of proteins with fluoresceinisocyanate altered the electrical charge of the conjugate sufficiently to make it combine in a salt-like binding with the cytoplasmic proteins of non-neoplastic epithelium The cytoplasmic proteins of neoplastic epithelium were believed to be less basic and therefore unable to combine with the relatively acid protein complex used as a stain If this assumption is rrect the pH at which the staining is performed may be of decisive nportance

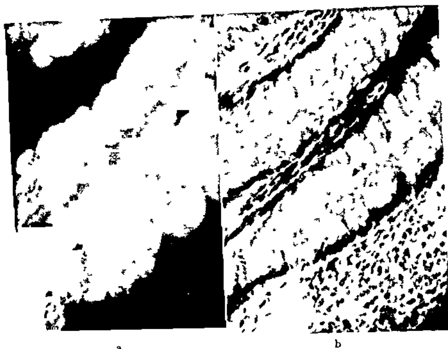
The results of *Hughes & Louis* however, have not been generally accepted *Nairn et al* (11) always found some fluorescence in cancer cells s well and could obtain no consistent absolute difference between noral and neoplastic cells

A factor which might conceivably affect the staining is the affinity hat free fluoresceinisocyanate has to mucinous substances Since the mount of mucins is certainly not the same in normal and neoplastic pithelium variations in the content of mucin could at least partially xplain any differences This source of possible error does not seem to ave been systematically investigated Furthermore [illegible]

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Fig 1a Normal colon epithelium stained with FTC-ovalbumin at pH 5 The cyto plasm fluoresces brightly the cells resembling 'corn on the cob' (× 500)
Fig 1b The same area as in Fig 1a after staining with PAS

with previous findings (10) a high concentration of formalin was found to abolish staining altogether Absolute methanol gave satisfactory results with the fluorescent stain but the PAS stain gave more consistent results and the sections did not tend to loosen from the glass if formalin was added to the methanol For these reasons a mixture of 4 parts of methanol and 1 part of 40 per cent formalin was found most suitable and was accordingly used in the main experiments

Autofluorescence in unstained sections A strong yellow white auto fluorescence emanating from the elastica of the vessels and a very faint blue-green autofluorescence in the normal mucosa made orientation possible After fixation, cells with a blue white, yellow green, or sometimes faintly red brown fluorescence were seen in all layers of the bowel wall Most of these corresponded to mast cells and histiocytes some were probably leucocytes Within cancerous areas no distinct structures could be discerned however, in regions where the cancer produced mucus a blue-white indistinct autofluorescence was seen Sometimes brilliant granules were seen in such masses, which by after-staining were seen to correspond to necrotic cells No autofluorescence appeared which resembled the characteristic apple green colour of the FTC conjugate

Stained sections *Normal colon epithelium* Staining at *pH 3, 4,* and

The aim of the present report is to assess the reliability of the method when applied to human colon and to investigate the influence of pH on the staining, with special reference to the reactions of certain PAS-positive mucinous substances. Cancerous and precancerous conditions of the colon were studied, since such material was readily available and well suited to a study of staining reactions of mucin. Rather than trying to include many cases, the emphasis has been on examining each case extensively in order to determine the consistency of the results in sections from different parts of the same colon.

MATERIAL AND METHODS

Tissue specimens. Four surgical specimens from typical colon carcinomas and one colon removed from a patient with massive polyposis were used.[1] The specimens were brought to the laboratory *in toto* and pieces of about 4 × 4 × 2 mm were excised. They were immediately immersed in isopenthane kept at a temperature of about −80° C. As soon as the pieces were frozen, they were removed from the isopenthane, wiped off, and stored at −25° C.

A Pearse cryostate set at 6 μ was used for sectioning. After thawing, the sections were dried (fan) for 30 minutes at room temperature. They were then fixed in a mixture of 4 parts of methanol and 1 part of 40 per cent formalin for 5 minutes. Afterwards the sections were again dried (fan) for 15 minutes and stained 15 minutes in a moist chamber.

Thorough washing was essential for good results. In some experiments the sections were washed for 10–15 minutes in 3 changes of phosphate buffered saline. Even better results were obtained by washing in running tap water for 25 minutes. The latter procedure was therefore adopted as standard. Fixation, drying and washing were carried out at room temperature.

The sections were mounted in buffered glycerol (pH 7) and were usually examined on the same day. After 1 to 2 days, blurring tended to occur. After undergoing examination in the fluorescence microscope, the sections were after-stained with PAS.

Preparation of conjugate. To diminish the possibility of immunological reactions (13, 14) egg albumin (4) was used as the protein component of the conjugate. Conjugation with fluoresceinisothiocyanate (FITC) was performed according to *Rinderknecht*... *Riggs et al.* (12). Excess FITC, carbonate and bicarbonate ions were removed in one step by gel filtration (5) on a column of Sephadex G 25.[2] The column was eluted with physiological saline, and the coloured zone containing the egg albumin-FITC was collected. Aliquots of 0.5 ml of the eluate were mixed with 2.5 ml buffer. The following buffers were used: pH 3, 4, and 5: 0.2M Na_2HPO_4 – 0.1M citric acid; pH 6, 7, and 8: 0.2M Na_2HPO_4 – 0.2M KH_2PO_4; pH 9, 10 and 11: 0.5 Na_2CO_3 – 0.5M $NaHCO_3$. The nine solutions were allowed to stand 30 minutes and were then used for staining. After the above procedures, the protein concentration of the solution used for staining was about 0.3 per cent.

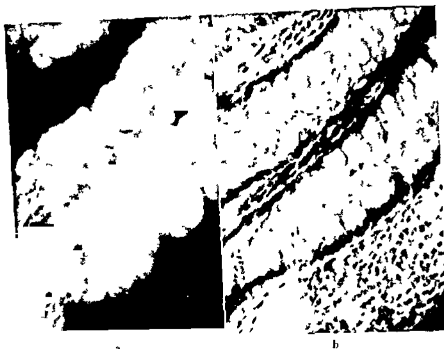
[illegible] Osram HBO 200
in the eyepiece
for black white

[1] By using the indicator system of the microscope, the same areas could be located and photographed in the after-stained sections.

RESULTS

Influence of fixation. In preliminary experiments different proportions of methanol and formalin were tried as fixatives. In accordance

1 The cooperation of the *Surgical Department of the University Hospital of Uppsala (Head: Professor Olle Hultén)* is gratefully acknowledged.
2 AB Pharmacia, Uppsala, Sweden.



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Stained sections Normal colon epithelium Staining at *pH 3, 4* and



Fig 2a Normal colon epithelium stained with FTC-ovalbumin at pH 6 The fluorescence outlines the cells giving the glands a netlike appearance (× 500)
Fig 2b The same area as in Fig 2a, after staining with PAS

5 gave an intense FTC-fluorescence in the epithelial cells The apple-green fluorescence was seen in the luminal and perinuclear parts of the cytoplasm, but not in the nuclei and along the cell membrane In this manner the cells came to look sharply demarcated from each other, resembling corn on the cob (Fig 1) Mucus on the mucosal surface and in the lumina of the glands also fluoresced brightly In general, all the structures that fluoresced were also PAS-positive

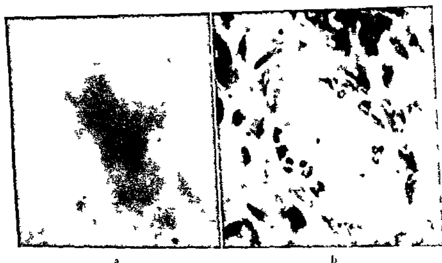
Staining at *pH 6* gave a completely different picture (Fig 2) Here the peripheral part of the cytoplasm and possibly also the cell membrane was intensely stained, whereas the PAS-positive parts hitherto stained at lower pH, remained unstained The glands in this manner came to have a net-like appearance At *higher pH* the result was principally the same, though there was a suggestion of an increased intensity at pH 7 and 8 and a decreasing intensity at even higher pH At pH 10 and 11 there was virtually no FTC-fluorescence left

The reactions described above were seen consistently in normal colon epithelium, irrespective of whether it was located in the immediate vicinity of cancerous or atypical glands or in completely unaffected parts of the colon

Free mesenchymal cells Mastcells were seen in great numbers, especially around the deeper portions of the mucosal glands Some of them



Fig 3
Mastcells in the deepest part of the mucosa showing enhanced auto fluorescence at pH 11 Some cells have ruptured and their spreading granulae fluoresce brilliantly (arrow) (× 500)



a b

Fig 4a Leucocytes in the submucosa Their cytoplasm stains well with FTC ovalbumin at pH 8 (× 500)
Fig 4b The same area as in Fig 4a stained with PAS

had released their granules to their surroundings The mast cells were strongly PAS positive but at no pH did they show any affinity to FTC conjugates The only pH induced difference was a slight enhancement of the brilliance of the autofluorescence at the higher pH (Fig 3)

Leucocytes were first distinguished at pH 6 when cytoplasmic apple green fluorescence of an intensity corresponding to that of the normal epithelium was seen (Fig 4) Cells belonging to this category were observed in the stroma of the mucosa and submucosa mainly around the vessels In some preparations an abundance of cells was seen in the

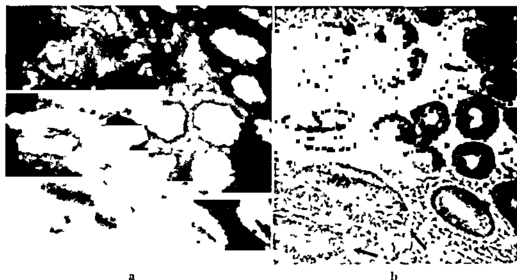


Fig 5a Colon carcinoma borderline stained with FTC-ovalbumin at pH 4 Normal epithelium stains as does PAS positive material in lumen of cancerous glands—cancer cells remain unstained (× 240)

Fig 5b The same area as in Fig 5, after staining with PAS Arrows indicate location of cancerous glands

muscular coat, judging from the appearance of the nuclei, these cells were eosinophils

Colon cancer Our cases were moderately differentiated carcinomas with an irregular production of mucus The individual cells were less distinctly demarcated from each other than the normal epithelial cells; their volume of cytoplasm was apparently diminished and they were seldom PAS-positive

At pH 3, 4, and 5 FTC-ovalbumin gave no fluorescence in the cancer cells (Fig 5) There was thus a considerable contrast between the stained normal epithelium and the unstained cancerous epithelium When the cancer areas contained mucinous substances, these had affinity to the conjugate which resulted in a mottled fluorescence which, in general, was parallel to the degree of PAS-positivity

At pH 6 the cancer cells remained unstained (Fig 6 and 7), provided the sections had been carefully prepared Artefacts in the form of a weak, diffuse, or sometimes granular FTC-fluorescence were sometimes seen together with brilliant masses of apple-green fluorescence in the marginal portions of lumina in the cancer vegetations Such artefacts also occurred outside the cancerous areas and sometimes even beside the sections These artefacts were either produced by inefficient washing or improper drying

With still increasing pH there was no change in the reactivity of the cancer cells Strongly fluorescing clumps corresponding to PAS-positive mucus were seen in the cancer vegetations The degree of fluorescence did not seem to be entirely due to the degree of PAS-positivity, since the presence of necrotic cells enhanced the apple-green fluorescence



Fig. 6a Normal colon glands stained with FTC ovalbumin at pH 6. Note typical fluorescence in cell cytoplasm giving glands a net like appearance (× 240)

Fig. 6b The same area as in Fig. 6a after staining with PAS

Fig. 6c Cancerous glands from another part in the same section. Note absence of fluorescence in the cells. The faint fluorescence seen is blue white autofluorescence from elastic elements (× 240)

Fig. 6d Same area as in Fig. 6c after staining with PAS

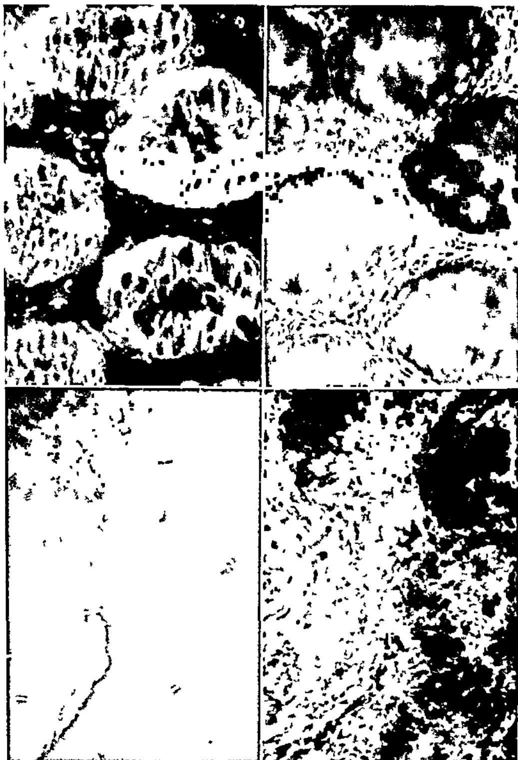


Fig 7a Normal colon glands stained with FITC ovalbumin at pH 7 Note typical fluorescence in the cytoplasm of the epithelium Leucocytes in the stroma also display cytoplasmic fluorescence (× 240)
Fig 7b Same area as in Fig 7a after staining with PAS
Fig 7c Cancerous glands from another part in the same section Note complete absence of any staining in the neoplastic epithelium Elastic tissue shows strong autofluorescence (× 240)
Fig 7d Same area as in Fig 7c after staining with PAS

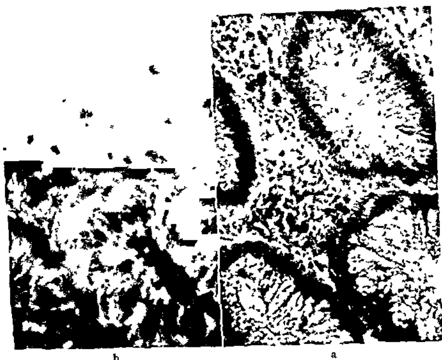


Fig 8a Normal epithelium adjacent to slightly atypical glands—a case of colon polyposis stained at pH 7 The atypical glands to the right show a definitely diminished fluorescence (× 600)
Fig 8b The same area after staining with PAS

without influencing the PAS positivity In fact, sometimes homogeneous PAS positive areas did not fluoresce at all The blue-white autofluorescence seen in the mucus of the cancer was less prominent at pH 6, 7 and 8, but increased simultaneously with a decrease of the secondary FTC fluorescence

Colon polyps with atypia Histologically normal glands showed the same fluorescence as glands from other preparations The intensity of the fluorescence was the same irrespective of whether the glands were bordered by atypical or normal epithelium

Some fluorescence was seen at pH 3 5 in the cytoplasm of the cells of the atypical glands Compared to normal epithelium, it was considerably weaker and often irregularly distributed Very often it was concentrated in the luminal portion of the cells In general, this phenomenon corresponded to PAS positive structures

Also at pH 6 and 7 some fluorescence was seen in the cytoplasm of atypical cells It was, however, distinctly weaker than in the histologically normal glands (Fig 8) With increasing pH the fluorescence diminished generally, and at pH 9 it was virtually abolished

The results have been summarized in Table 1 It is seen that a satisfactory differential staining was obtained especially at pH 7 and 8



Fig 7a Normal colon glands stained with FTC ovalbumin at pH 7 Note typical fluorescence in the cytoplasm of the epithelium Leucocytes in the stroma also display cytoplasmic fluorescence (× 240)

Fig 7b Same area as in Fig 7a after staining with PAS

Fig 7c Cancerous glands from another part in the same section Note complete absence of any staining in the neoplastic epithelium Elastic tissue shows strong autofluorescence (× 240)

Fig 7d Same area as in Fig 7c after staining with PAS

early work (3, 4), but it has only been generally accepted after the experimental work by *V Mayersbach* (10), *Curtain* (1) and *Goldstein et al* (2) When protein is coupled to fluorescein isothiocyanate, NH-groups are engaged and the conjugates get a larger negative charge In the neutral pH range, the relatively acid conjugate is assumed to form a salt like binding to the relatively basic proteins of the cytoplasm of a normal epithelial cell If the pH is lowered, the ionization of the carboxygroups is presumably depressed which will prevent any binding to the positively charged cytoplasmic proteins If the pH at which the staining reaction is performed is increased a change of the electrical charge of the cytoplasmic proteins (depression of the ionization of the aminogroups) is presumably responsible for the abolishment of the staining

The difference found between atypical but not yet invasive epithelium and normal epithelium is of considerable interest The difference was not absolute as in outright cancer, but nevertheless fully noticeable Since the cytoplasmic area of the normal cells was equal to or less than that of the atypical cells, the more intense brilliance in the former cells cannot be explained as due merely to a difference in the amount of cytoplasm Instead there seems to exist a real difference in the affinity to the FTC-conjugate No areas with a completely normal histology showed decreased fluorescence

Lagunoff et al (6) have described a method to visualize mastcells by treating frozen dried sections by formalin vapour, a procedure which induced a yellow green fluorescence These authors manitained that the fluorescence indicated a reaction product between histamine and formaldehyde The fluorescence which we observed after fixation by formalin methanol in mast cells seemed to be of a similar nature and would thus perhaps constitute a histochemical test for histamine The intensity of the fluorescence increased at higher pH

A major result of this study was the reproducibility of the staining reactions Once a proper, standardized technique was adopted, the cells belonging to different categories reacted in a fully predictable manner Large areas of atypical or cancerous areas were scanned and mapped out by putting series of photomicrographs together and by comparing these maps with the results of the after-staining it could be established that irrespective of where or how they were growing, viable cancer cells invariably failed to stain at any pH from 3–11

SUMMARY

Sections from normal colon, colon polyps and colon [illegible]

not at

At p[illegible] [illegible] fluoresceinisothiocyanate complex stained

between neoplastic and normal epithelium It is also seen that the conjugate served as an excellent stain for mucin in epithelium at pH 3 5 With regard to the epithelium, the staining at these acid pH values was essentially similar to PAS stain That PAS-positivity *per se* did not necessarily mean a positive FTC-ovalbumin stain was shown by results with mast cells, which were strongly PAS-positive, but nevertheless had no affinity for the fluorescent conjugate

TABLE 1

The Staining Properties of Fluoresceinisothiocyanate-Ovalbumin at Different Levels of pH

		FTC ovalbumin pH								
		3	4	5	6	7	8	9	10	11
Normal epithelium		—	—	-	+	++	++	(+)	—	—
Atypical* epithelium		—	—	—	(+)	(+)	(+)	(+)	—	—
Malignant epithelium		—	—	—	—	—	—	—	—	—
PAS positive	Leucocytes	—	—	—	+	++	++	(+)	—	—
	Mast cells	—	—	—	—	—	—	—	—	—
	Mucin§	+++	+++	+++	(+)	—	—	—	—	—

* *i e*, epithelium from polyps with atypia but no definite infiltrative growth
§ Extracellular and intracellular in epithelial cells

The mechanism behind the staining of mucus was further investigated Theoretically it was possible that the result at an acid pH was dependent on a liberation of FTC from the protein, since free FTC gave essentially the same result as the conjugate did at pH 3 5 However, this was apparently not the case since a rerun of conjugate on a Sephadex column uniformly showed only one zone corresponding to the protein-FTC complex and no trace of any unbound FTC Prolonged dialysis also failed to reveal the liberation of any free FTC at an acid pH From this it would seem probable that other radicals in the FTC-molecules than those engaged in the binding to the albumin were responsible for the binding to the epithelial mucin

DISCUSSION

Our results have mainly confirmed those of *Louis* (8) in showing an apparently absolute and characteristic difference in the staining reaction between cancer cells and normal cells in the human colon Since we have used ovalbumin as the protein moiety of the staining complex, the results cannot be explained on the basis of any antigen-antibody reactions (13, 14) The influence of the pH on the outcome of the staining is in agreement with what might be expected from what is known or assumed about the interaction of the protein-FTC complex and the cell cytoplasm It seems to be well substantiated that the main part of the "non-specific" staining that occurs after FTC-protein staining depends on salt-like bindings between cytoplasmic protein and the conjugate *Hughes & Louis* postulated this kind of mechanism in their

early work (3, 4), but it has only been generally accepted after the experimental work by *V Mayersbach* (10), *Curtain* (1) and *Goldstein et al* (2) When protein is coupled to fluorescein isothiocyanate, NH_2-groups are engaged and the conjugates get a larger negative charge In the neutral pH range, the relatively acid conjugate is assumed to form a salt like binding to the relatively basic proteins of the cytoplasm of a normal epithelial cell If the pH is lowered, the ionization of the carboxygroups is presumably depressed which will prevent any binding to the positively charged cytoplasmic proteins If the pH at which the staining reaction is performed is increased a change of the electrical charge of the cytoplasmic proteins (depression of the ionization of the amino groups) is presumably responsible for the abolishment of the staining

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SUMMARY

Sections from normal colon colon polyps and colon carcinoma have been stained with egg albumin fluoresceinisothiocyanate conjugates at pH 3 11 At pH 7–8 the cytoplasm of normal epithelium stained strongly the polyp epithelium stained very weakly, and cancerous epithelium not at all These findings confirm those of *Hughes, King & Louis*

At pH 3 5 the egg albumin fluoresceinisothiocyanate complex stained

epithelial mucins very intensively, paralleling the intensity of a PAS-stain At the same pH, mast cells were left unstained, although they were strongly PAS-positive Neutrophilic and eosinophilic leucocytes stained strongly at pH 7–8 No structures were stained at pH 10–11

At an acid pH the egg albumin-fluoresceinisothiocyanate method provided a valuable stain for epithelial mucin At neutral or slightly alkaline pH the stain clearly differentiated between neoplastic and normal epithelial cells in the colon

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FROM CANCERREGISTERET AND DEPARTMENT OF PATHOLOGY, FINSEN INSTITUTE COPENHAGEN DIRECTOR JOHANNES CLEMMESEN M D

STUDIES ON THE NEWT TEST FOR CARCINOGENICITY

4 *Supplementary Experiments on Specificity*

By

E. ARFFMANN

Received 11 xi 63

In a previous report (*Arffmann* 1963) the specificity of the newt test was studied by means of non-carcinogenic and weak carcinogenic hydrocarbons The results were similar to those known from experiments on mammals A few exceptions, however, necessitate some supplementary experiments, and on the other hand the positive conclusion as to polycyclic hydrocarbons encourages to test some carcinogens of a different chemical structure

The results of *Sorensen* (1962) confirming the ability of arsenic trioxide to induce glycolysis in Earle cells and thus change the metabolism *in the direction* of malignancy, made it natural to test this substance simultaneously in newts In these experiments isonicotinic acid hydrazide (*isoniazid*) was included, a water-soluble compound, to which reports of later years have ascribed a carcinogenic potency

The carcinogenicity of alkylating agents has been studied extensively during recent years Among these substances, as suggested to us by Professor H Druckrey, it was especially interesting to compare the effect of diethylnitrosamine and N-nitroso N-methyl urethane, the first supposedly becoming active only after metabolism especially in the liver, the latter having a direct, local effect

Finally a few experiments on Danish salamanders (Triton vulgaris) are included

TECHNIQUE

In the experiments with hydrocarbons the usual technical procedures were applied (*Arffmann* 1963)

Arsenic trioxide was found weakly soluble in destilled water In spite of vigorous stirring and incubation at 37° C a 10^{-3} *molar* solution kept a white sediment which however was considerably reduced by the treatment Half of the animals were injected with the clear solution and the others with the solution following an attempt of suspending the small sediment by shaking

In the first experiments with arsenic trioxide in peanut oil the 0 1 per cent (w/v) suspension was heated at 100° C for 15 minutes like the other test objects The 0 5 per cent suspensions were like the isoniazid suspensions prepared in the dispensary of the Finsen Institute using simple pharmaceutical procedures

The authors thanks are due to *Professor H Druckrey* Freiburg for suggesting the study of diethylnitrosamine and N nitroso N methyl urethane and for supplying these compounds

Diethylnitrosamine was easily dissolved in destilled water and peanut oil in the applied concentrations (v/v), and so was N nitroso N methyl urethane in the oil while this compound is insoluble in water The injected volume of diethylnitrosamine in water was raised to 0 05–0 09 cc

Further technical procedures have previously been described and were used unaltered on Triton vulgaris

RESULTS

The *supplementary experiments with non-carcinogenic and carcinogenic* hydrocarbons are shown in Tables 1–3 The inactivity of anthracene in 0 1 and 0 5 per cent solution and of triphenylene in 0 5 per cent solution is confirmed in experiments nos 50 and 62 (Table 1), most plainly in the latter, the first experiment being impaired by the weak reaction to benzpyrene This phenomenon reflects a seasonal variation in sensitiveness of the newt test, the animals being less reactive in early spring, when secondary sex characteristics are most prominent The coincidence of resistance and sexual development makes it probable that the low epidermal reactivity is due to an altered hormonal balance The phenomenon was already observed in a few earlier experiments (*Arffmann* 1963, Table 3, exper no 27–29), in which the reaction to chrysene was remarkably weak, and has also been described by *Seilern Aspang & Kratochwil* 1962)

The weakly carcinogenic 1,2,4 trimethylphenanthrene gave no epithelial response in 0 1 and 0 5 per cent solution, and experiment no 60 (Table 1) shows that an increase of the concentration to 1 per cent does not change this result The positive reaction in all 8 animals injected with 1 per cent of benzpyrene forms a distinct contrast

In previous experiments 3′,6-dimethyl benz[*a*]anthracene was found to act in the newt like a strong carcinogen A reduction in the concentration might, however, reveal a difference between this substance, supposed to be non-carcinogenic, and the strongly active dibenz[*a,h*]-anthracene Table 2 shows this to be the case In 0 025 per cent, 0 0125 per cent, and 0 00625 per cent solution the activity of 3′,6-dimethyl benz[*a*]anthracene is remarkably reduced and is distinctly lower than that of dibenzanthracene in the same concentrations

Previous results (*Arffmann* 1963) indicated that the degree of response parallels the concentration of the test solution Experiments with strong carcinogens supported this view, and the present report extends the experience on weak carcinogens (Table 3) The experiments were carried out in the autumn and so, according to the seasonal variation, the reaction to the tested substances is relatively strong The many positive reactions to the weakest solutions and the two positive controls invariably veils the differences, but nevertheless the incidence of positive responses increases with the concentration of the carcinogens This is most apparent in the case of benz[*a*]anthracene, but also applies to chrysene

Arsenic trioxide had no effect in watery solution, while injection of

TABLE 1

Supplementary Experiments with Anthracene, Triphenylene and 1,2,4-Trimethylphenanthrene

Experiment No	50									
Substance	Soya bean oil		Anthracene 0.1% in soya bean oil		Anthracene 0.5% in soya bean oil		Triphenylene 0.5% in soya bean oil		Benzo[a]pyrene 0.1% in soya bean oil	
Site of application	Tail									
Number of animals (Triton cristatus)	8		8		8		8		8	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on 4-6 day										–
9			–		–	–	–		–	–
12-13	–	–		–		–	–	–	=	
15			–	–	–	–			–	(+)
18	–	–	–	–	–	–	–			–
Animals died during experiment	0		0		0		1		1 (included)	

Experiment No	62									
Substance	Soya bean oil		Anthracene 0.1% in soya bean oil		Anthracene 0.5% in soya bean oil		Triphenylene 0.5% in soya bean oil		Dibenz[a,h]anthracene 0.1% in soya bean oil	
Site of application	Tail									
Number of animals (Triton cristatus)	8		8		8		8		9	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on 4-6 day					–					
9			–	–		–	–	–	+	
12-13		–	–	–		–	–	–	(+)	+
15	–		–	–	–	–	–	–	++	–
18	–	–	–			–	–	–	–	±
Animals died during experiment	0		1 (included)		1 (included)		0		1	

Experiment No	60					
Substance	Peanut oil		1,2,4-Trimethylphenanthrene 1% in peanut oil		Benzo[a]pyrene 1% in peanut oil	
Site of application	Tail					
Number of animals (Triton cristatus)	4		8		8	
Sex	♂	♀	♂	♀	♂	♀
Epidermal reaction on 4-6 day						
9	–		–	–	+	+
12-13			–	–	+	+
15	–		–	–	+	+
18		–	–	–	+	+
Animals died during experiment	0		0		0	

Diethylnitrosamine was easily dissolved in destilled water and peanut oil in the applied concentrations (v/v), and so was N-nitroso-N-methyl urethane in the oil while this compound is insoluble in water. The injected volume of diethylnitrosamine in water was raised to 0.05–0.09 cc.

Further technical procedures have previously been described and were used unaltered on Triton vulgaris.

RESULTS

The supplementary experiments with non-carcinogenic and carcinogenic hydrocarbons are shown in Tables 1–3. The inactivity of anthracene in 0.1 and 0.5 per cent solution and of triphenylene in 0.5 per cent solution is confirmed in experiments nos. 50 and 62 (Table 1), most plainly in the latter, the first experiment being impaired by the weak reaction to benzpyrene. This phenomenon reflects a seasonal variation in sensitiveness of the newt test, the animals being less reactive in early spring, when secondary sex characteristics are most prominent. The coincidence of resistance and sexual development makes it probable that the low epidermal reactivity is due to an altered hormonal balance. The phenomenon was already observed in a few earlier experiments (*Arffmann* 1963, Table 3, exper. no. 27–29), in which the reaction to chrysene was remarkably weak, and has also been described by *Seilern-Aspang & Kratochwil* 1962).

The weakly carcinogenic 1,2,4-trimethylphenanthrene gave no epithelial response in 0.1 and 0.5 per cent solution, and experiment no. 60 (Table 1) shows that an increase of the concentration to 1 per cent does not change this result. The positive reaction in all 8 animals injected with 1 per cent of benzpyrene forms a distinct contrast.

In previous experiments 3',6-dimethyl benz[a]anthracene was found to act in the newt like a strong carcinogen. A reduction in the concentration might, however, reveal a difference between this substance, supposed to be non-carcinogenic, and the strongly active dibenz[a,h]-anthracene. Table 2 shows this to be the case. In 0.025 per cent, 0.0125 per cent, and 0.00625 per cent solution the activity of 3',6-dimethyl benz[a]anthracene is remarkably reduced and is distinctly lower than that of dibenzanthracene in the same concentrations.

Previous results (*Arffmann* 1963) indicated that the degree of response parallels the concentration of the test solution. Experiments with strong carcinogens supported this view, and the present report extends the experience on weak carcinogens (Table 3). The experiments were carried out in the autumn and so, according to the seasonal variation, the reaction to the tested substances is relatively strong. The many positive reactions to the weakest solutions and the two positive controls invariably veils the differences, but nevertheless the incidence of positive responses increases with the concentration of the carcinogens. This is most apparent in the case of benz[a]anthracene, but also applies to chrysene.

Arsenic trioxide had no effect in watery solution, while injection of

TABLE 1

Supplementary Experiments with Anthracene, Triphenylene and 1,2,4-Trimethylphenanthrene

Experiment No.	50					62					60		
Substance	Soya bean oil	Anthracene 0.1% in soya bean oil	Anthracene 0.5% in soya bean oil	Triphenylene 0.5% in soya bean oil	Benzopyrene 0.1% in soya bean oil	Soya bean oil	Anthracene 0.1% in soya bean oil	Anthracene 0.5% in soya bean oil	Triphenylene 0.5% in soya bean oil	Dibenz[a,h]anthracene 0.1% in soya bean oil	Peanut oil	1,2,4-Trimethylphenanthrene 1% in peanut oil	Benzo[a]pyrene 1% in peanut oil
Site of application	Tail					Tail					Tail		
Number of animals (Trit. meristatus)	8	8	8	8	8	8	8	8	8	9	4	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal reaction													
0 1													
4 6 day					–			–					
9		–	–	–			–	–	– –	+	–	– –	+ +
12 13	–	–	–	–	=	– –	– –	–	– –	(+) +		–	+ +
15	–	– –	–		– (+)	–	–	– –	–	++ –	–	– –	+ +
18	–	–	–	–		–	–	–	– –	– ±	–	– –	+ +
Animals died during experiment	0	0	0	1	1 (included)	0	1 (included)	1 (included)	0	1	0	0	0

Diethylnitrosamine was easily dissolved in destilled water and peanut oil in the applied concentrations (v/v), and so was N nitroso N methyl urethane in the oil while this compound is insoluble in water The injected volume of diethylnitrosamine in water was raised to 0 05 0 09 cc

Further technical procedures have previously been described and were used unaltered on Triton vulgaris

RESULTS

The supplementary experiments with non-carcinogenic and carcinogenic hydrocarbons are shown in Tables 1 3 The inactivity of anthracene in *0 1 and 0 5 per cent solution* and of triphenylene in *0 5* per cent solution is confirmed in experiments nos 50 and 62 (Table 1), most plainly in the latter, the first experiment being impaired by the weak reaction to benzpyrene This phenomenon reflects a seasonal variation in sensitiveness of the newt test, the animals being less reactive in early spring, when secondary sex characteristics are most prominent The coincidence of resistance and sexual development makes it probable that the low epidermal reactivity is due to an altered hormonal balance The phenomenon was already observed in a few earlier experiments (*Arffmann* 1963, Table 3, exper no 27–29), in which the reaction to chrysene was remarkably weak, and has also been described by *Seilern Aspang & Kratochwil* 1962)

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Previous results (*Arffmann* 1963) indicated that the degree of response parallels the concentration of the test solution Experiments with strong carcinogens supported this view, and the present report extends the experience on weak carcinogens (Table 3) The experiments were carried out in the autumn and so, according to the seasonal variation, the reaction to the tested substances is relatively strong The many positive reactions to the weakest solutions and the two positive controls invariably veils the differences, but nevertheless the incidence of positive responses increases with the concentration of the carcinogens This is most apparent in the case of benz[*a*]anthracene but also applies to chrysene

Arsenic trioxide had no effect in watery solution, while injection of

TABLE 3

Experiments with Varying Concentrations of the [illegible] Carcinogens

Experiment No.	63																	
Substance	Peanut oil		Benzanthracene 0.[illegible] % in peanut oil		Benzanthracene 0.1 % in peanut oil		Benzanthracene [illegible] % in peanut oil		Chrysene 0.[illegible] % in peanut oil		Chrysene 0.1 % in peanut oil		Chrysene 0.[illegible] % in peanut oil		Benzopyrene 0.5 % in peanut oil		Benzopyrene 1 % in peanut oil	
Site of application	Tail																	
Number of animals (for each sex)	8		8		8		8		8		8		8		8		11	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on 7 th day																		
9			+		+					−	+	+		+	+	+	+	+(+)
12						+		++		++		+		++		++		++(+)
15	−		+	+	(+)		+	+	+		+	+	+	+	+	+	+	++
18			+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Animals [illegible] during experiment	0		0		0		1 ([illegible])		0		0		0		0		0	

Experiment No.	64															
Substance	Peanut oil		Benzanthracene 0.5 % in peanut oil		Benzanthracene 0.1 % in peanut oil		Benzanthracene 0.[illegible] % in peanut oil		Chrysene 0.05 % in peanut oil		Chrysene 0.1 % in peanut oil		Chrysene 0.5 % in peanut oil		Dibenzanthracene 0.5 % in peanut oil	
Site of application	[illegible]															
Number of animals (for each sex)	8		8		8		8		8		8		8		8	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on 7 th day								(+)				−				
9					+			+	(+)			+			+	−
12				+		++	−	+		∓	+	+		++		++
15	+			+		+	+	(+)	+	+	+	+	+	+	+	+
18	+		+	+			+			+	+		+	+	+	+
Animals [illegible] during experiment	0		0		2 ([illegible])		1 ([illegible])		0		1 ([illegible])		0		0	

TABLE 2

Experiments with 3,6 Dimethyl benz[a]anthracene (DMBA) and Dibenz[a,h]anthracene (DBA) in Descending Concentrations

Experiment No	57			58			9		
Substance	Soya bean oil	DMBA 0.025% in soya bean oil	DBA 0.025% in soya bean oil	Peanut oil	DMBA 0.0125% in peanut oil	DBA 0.0125% in peanut oil	Peanut oil	DMBA 0.00625% in peanut oil	DBA 0.00625% in peanut oil
Site of application	Tail			Tail			Tail		
Number of animals (Triton cristatus)	8	8	8	8	8	8	8	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal reaction on									
9 day		−	+ +		−	− +	−	− −	−
12	− −	− −	+ +	−	+ −	+		+ −	+ +
15	− −	+ −	+ +	−	+	+		+ −	+ +
18	− −	− +	+		− −	+ −		− −	+ −
Animals died during experiment	0	0	0	1	0	0	0	0	0

oily suspensions resulted in a few positive or partly positive reactions (Table 4) The number of these, however, was mostly equal to that seen in controls and distinctly lower than the number of epithelial reactions in animals injected with carcinogenic hydrocarbons The faintly positive result in experiment no 55 is questionable, since it comprised only one case of hyperplasia and one doubtful response

In two out of three experiments isoniazid induced a weakly positive response, equal to, or less marked than that to chrysene (Table 4)

No reaction was seen to diethylnitrosamine in a 0 5 per cent watery solution, while change of the vehicle to peanut oil revealed an activity of the same order as that exhibited by 0 5 per cent N-nitroso-N-methyl-urethane (Table 5) (Figs 1 and 2) Experiment no 60 using 1 per cent solutions strongly suggests, however, that N-nitroso-N methyl-urethane is a more potent carcinogen than diethylnitrosamine Both alkylating agents were distinctly less active than benzpyrene and dibenzanthracene

In 10 per cent solution N-nitroso N-methyl-urethane showed a local toxic effect causing necrosis in two animals and pronounced oedema in several others In one salamander the necrosis was so extensive that the case had to be discarded The toxicity may have inhibited the epithelial response in this experiment Reduction in concentration was

n l Isonicot nic Aci l Hy lra i le (INH)

[illegible]			52				53				55	
Peanut oil	INH in peanut oil (0 5% suspen s on	Chr sene 0 5% in peanut o l	Peanut o l	INH in peanut o l (0 5% suspe s on)	As₂O in peanut o l (0 5% suspen s on)	Chrysene 0 5% in peanut o l	Peanut oil	INH in peanut o l (0 5% susp n s on)	As₂O₃ in peanut o l (0 5% suspen s on)	MCA 0 1% in peanut o l	Peanut o l	As₂O₃ in peanut o l (0 5% suspen s on)
Part of ta l prox n al to secundary ta l			Ta l				Tail				Ta l	
3	6	5	8	8	8	8	8	8	8	8	8	8
♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
−	+	+		−		( ) +	+	(+)	−	(+) ( ) + +	− − − −	( ) + − −
1 in cluded	1 in cluded	1 in clude l	2 1 in cluded	0	0	0	0	0	1	0	0	1 (in clude l)

DISCUSSION

The repeated negative results with anthracene at 0 1 and 0 5 per cent and with triphenylene at 0 5 per cent make it reasonable to assume that the earlier discussed positive reactions to these compounds in one experiment (*Arffmann* 1963 Table 7) were due to the influence of skin infection and general disease In another experiment (*Arffmann* 1963 Table 4 exper no 38) the positive reaction to anthracene was confined to one animal and so it may have been unspecific Thus the accordance with results in mammals was reestablished for simple non carcinogenic hydrocarbons

The present experiments with 3 6 dimethyl benz[*a*]anthracene (Table 2) show that it is possible with the newt test to distinguish quantitatively between the complex "non-carcinogenic" hydrocarbon and the closely related dibenz *a h*]anthracene The reaction to 3,6 dimethyl benz[*a*]-anthracene however is still positive even at the low concentrations and

T[illegible]

Experiments with Arsenic Trioxide (A[illegible]

Experiment No	17				18						19				
Substance	Dest water		As_2O_3 in dest water (10^{-3}M solution)		Peanut oil		As_2O_3 in peanut oil (0 1% suspension)		DBA* 0 1% in peanut oil		Peanut oil		As_2O_3 in peanut oil (0 1% suspension)		[D... 0... pe...]
Site of application	Tail				Tail						Tail				
Number of animals (Triton cristatus)	8		8		10		10		10		10		10		
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Epidermal reaction on															
3 6 day		–				–				–		=		–	+
7 9 -	–		–		–		–								
10 12	–	–					–	–	+	+					+
11 15					–	+	+	–	+	+	–	–	–	(	+
18 20				–	+	–		(+)		+		(+)			+'
42						–			–	(+)					
Animals died during experiment	1 (in cluded)		0		1 (in cluded)		2		1 (in cluded)		5 (2 in cluded)		5 (3 in cluded)		(1 clu...

* DBA = Dibenz a h]anthracene
§ MCA — 3 Methylcholanthrene
† Sections technically bad

followed by a decrease of toxic symptoms, which were slight at 1 per cent and had disappeared at 0 5 per cent Diethylnitrosamine showed no local toxicity In one experiment (no 61) specimens were taken from the liver of all but 4 animals injected with diethylnitrosamine and, for control, N-nitroso-N-methyl urethane, but histological examination revealed no degenerative lesions of the parenchyma

The positive controls in experiment no 61 were injected with 0 5 per cent benzpyrene in the midline of the back The strongly positive result confirms the previous observation in one case (see *Arffmann* 1963, Fig 5) that application of the carcinogen in this regeneratory zone is more effective than injection in the neutral area of the back

Table 6 shows results obtained in Triton vulgaris by the use of two strong and one weak carcinogenic hydrocarbons The animals reacted in the same way as Triton cristatus (Fig 3) and were even more sensitive to the carcinogens Epidermal hyperplasia was seen in one control animal

4

and Isonicotinic Acid Hydrazide (INH)

46			5[illegible]				53				5[illegible]	
Peanut oil	INH in peanut oil (0.5%, suspension)	Chrysene 0.5% in peanut oil	Peanut oil	INH in peanut oil (0.5%, suspension)	As_2O_3 in peanut oil (0.5%, suspension)	Chrysene 0.5% in peanut oil	Peanut oil	INH in peanut oil (0.5%, suspension)	As_2O_3 in peanut oil (0.5%, suspension)	MCA 0.1% in peanut oil	Peanut oil	As_2O_3 in peanut oil (0.5%, suspension)
Part of tail proximal to secondary tail			Tail				Tail				Tail	
3	6	5	8	8	8	8	8	8	8	8	8	8
♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
											−	
		−		−			−	−	− −	(+)		(±)
−	+	−		− −		( )		(+)		(+) +	−	+
				− −	+	−	− +				− −	−
		+		−		+ −	−	−		+	−	− −
1 (included)	1 (included)	1 (included)	2 (1 included)	0	0	0	0	0	1	0	0	1 (included)

DISCUSSION

The repeated negative results with anthracene at 0.1 and 0.5 per cent and with triphenylene at 0.5 per cent make it reasonable to assume that the earlier discussed positive reactions to these compounds in one experiment (*Arffmann* 1963, Table 7) were due to the influence of skin infection and general disease. In another experiment (*Arffmann* 1963, Table 4, exper. no. 38) the positive reaction to anthracene was confined to one animal and so it may have been unspecific. Thus the accordance with results in mammals was reestablished for simple, non carcinogenic hydrocarbons.

The present experiments with 3,6-dimethyl benz[*a*]anthracene (Table 2) show that it is possible with the newt test to distinguish quantitatively between the complex "non-carcinogenic" hydrocarbon and the closely related dibenz[*a,h*]anthracene. The reaction to 3,6-dimethyl benz[*a*]-anthracene, however, is still positive even at the low concentrations and

Experiments with Arsenic Trioxide (

Experiment No	17				18						19			
Substance	Dest water		As_2O_3 in dest water (10^{-3}M solution)		Peanut oil		As_2O_3 in peanut oil (0.1% suspension)		DBA* 0.1% in peanut oil		Peanut oil		As_2O_3 in peanut oil (0.1% suspension)	
Site of application	Tail				Tail						Tail			
Number of animals (Triton cristatus)	8		8		10		10		10		10		10	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Epidermal reaction on														
3–6 day		–				–						–		–
7–9	–		–		–									
10–12	–	–			–	–	–		+	+		–	–	
14–15			–		–	+	+	–	+	+		–	–	(
18–20					+	–		(+)		+		(∓)		
42						–				(+)				
Animals died during experiment	1 (included)		0		1 (included)		2		1 (included)		3 (2 included)		3 (3 included)	

* DBA = Dibenz a h anthracene
§ MCA = 3 Methylcholanthrene
† Sections technically bad

followed by a decrease of toxic symptoms, which were slight at 1 per cent and had disappeared at 0.5 per cent. Diethylnitrosamine showed no local toxicity. In one experiment (no. 61) specimens were taken from the liver of all but 4 animals injected with diethylnitrosamine and, for control, N-nitroso-N-methyl-urethane, but histological examination revealed no degenerative lesions of the parenchyma.

The positive controls in experiment no. 61 were injected with 0.5 per cent benzpyrene in the midline of the back. The strongly positive result confirms the previous observation in one case (see *Arffmann* 1963, Fig. 5) that application of the carcinogen in this regeneratory zone is more effective than injection in the neutral area of the back.

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n l Ison cot n c Aci l Hydra i le (INH)

46				52			53				55	
Peanut oil	INH in peanut oil (0 5% suspension)	Chrysene 0 5% in peanut oil	Peanut oil	INH in peanut oil (0 5% suspension)	As O_3 in peanut oil (0 5% suspension)	Chrysene 0 5% in peanut oil	Peanut oil	INH in peanut oil (0 5% suspension)	As_2O_3 in peanut oil (0 5% suspension)	MCA 0 1% in peanut oil	Peanut oil	As_2O_3 in peanut oil (0 5% suspension)
Part of tail proximal to secondary tail			Tail				Tail				Tail	
3	6	5	8	8	8	8	8	8	8	8	8	8
♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
				−	−			−	−	(+)	−	( )
	+				−	( )	−	(+)		( ) +	−	+
							+			−		
						+ +				+	−	−
1 (included)	1 (included)	1 (included)	2 (1 included)	0	0	0	0	0	1	0	0	1 (included)

DISCUSSION

The repeated negative results with anthracene at 0 1 and 0 5 per cent and with triphenylene at 0 5 per cent make it reasonable to assume that the earlier discussed positive reactions to these compounds in one experiment (*Arffmann* 1963 Table 7) were due to the influence of skin infection and general disease In another experiment (*Arffmann* 1963 Table 4 exper no 38) the positive reaction to anthracene was confined to one animal and so it may have been unspecific Thus the accordance with results in mammals was reestablished for simple, non carcinogenic hydrocarbons

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TABLE 5

Experiments with Diethylnitrosamine (DENA) and N-nitroso-N-methyl-urethane (NMU)

Experiment No	57			60				61				
Substance	Soya-bean oil	NMU 10% in soya bean oil	DBA* 0.025% in soya bean oil	Peanut oil	DENA 1% in peanut oil	NMU 1% in peanut oil	BP§ 1% in peanut oil	Peanut oil	DENA 0.5% in dest water	DENA 0.5% in peanut oil	NMU 0.5% in peanut oil	BP§ 0.5% in peanut oil
Site of application	Tail			Tail				Tail				Back
Number of animals (Triton cristatus)	8	8	8	4	8	8	8	4	8	8	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal reaction on												
9 day	− −	+ (+)	+ +	−	− (+)	(+) (+)	+ +	−	− −	+ −	− −	+ +
12	−	− (+)	+ +	−	+ −	+ (+)	+ +	−	− −	+ −	− +	− +
15	−	(+)	+ +	−	− −	− (+)	+ +	−	− −	+ −	(+) −	+ +
18	− −	− †	+ −	−	− −	+ (+)	+ +	−	− −	− −	(+) −	+ (+)
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0

* DBA = Dibenz[a h]anthracene
§ BP = Benzo[a]pyrene
† Necrosis

TABLE 6

Experiments on Danish Salamanders (Triton vulgaris)

Experiment No	53		56				61	
Substance	3-Methylcholanthrene 0.1% in peanut oil	3-Methylcholanthrene 0.1% in peanut oil	Peanut oil	Peanut oil	Benzo[a]pyrene 0.5% in peanut oil	Benzo[a]pyrene 0.5% in peanut oil	Peanut oil	Chrysene 0.1% in peanut oil
Site of application	Tail		Tail				Tail	
Number of animals	8 (Triton crist.)	8 (Triton vulg.)	4 (Triton crist.)	4 (Triton vulg.)	4 (Triton crist.)	4 (Triton vulg.)	3 (Triton vulg.)	4 (Triton vulg.)
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on								
5 day							—	
9	(+) —	+	—		—	+		
12 13	(+) +	+ +		—		+	(+)	+ +
15	—	+ +		—	+	+	—	(+) +
18	+ +	( ) +			+	+		
Animals died during experiment	0	0	0	0	0	0	1 (included)	0

so the compound qualitatively has to be numbered among substances active in the newt.

Experiment no. 60 (Table 1) confirms the inactivity of 1,2,4-trimethylphenanthrene. This disagreement with results in mice has been discussed earlier.

The incidence of positive reactions after injections of carcinogenic hydrocarbons appears to be slightly higher in female than in male newts, especially when weak carcinogens are used, but the difference between the two sexes is not significant.

In his large review on arsenical cancer *Neubauer* (1947) pointed out that few tumours have been induced in experimental animals by any type of application. Arsenic alone gave but doubtful results. This statement corresponds to the negative outcome of our testing in the newt, in contrast to *Sørensen's* results (1962) *in vitro*.

Carcinogenicity of isonicotinic acid hydrazide has been shown by several authors (*Juhász et al.* 1957, *Mori et al.* 1959 and 1960, *Schwan* 1961, *Biancifiori & Ribacchi* 1962). The compound was applied peror-

TABLE 5

Experiments with Diethylnitrosamine (DENA) an … N methyl urethane (NMU)

Experiment No	57				60				61			
Substance	Soya bean oil	NMU 10% in soya bean oil	DBA* 0.025% in soya bean oil	Peanut oil	DENA 1% in peanut oil	NMU 1% in peanut oil	BPS 1% in peanut oil	Peanut oil	DENA 0.5% in dest water	DENA 0.5% in peanut oil	NMU 0.5% in peanut oil	BPS 0.5% in peanut oil
Site of application	Tail			Tail				Tail				Back
Number of animals (Triton cristatus)	8	8	8	4	8	8	8	4	8	8	8	8
Sex	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Epidermal reaction on												
9 day	– –	+ (+)	+ +	–	– (+)	(+) (+)	+ +	–	– –	+ –	– –	+ +
12	–	– (+)	+ +	–	+ –	+ (+)	+ +	–	– –	+ –	– +	– +
15	– –	– (+)	+ +	–	– –	– (+)	+ +	–	– –	+ –	(+) –	+ +
18	– –	– †	+ –	–	– –	+ (+)	+ +	–	– –	–	(+) –	+ (+)
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0

* DBA = Dibenz a h anthracene
§ BP = Benzo a pyrene
† Necrosis

ally or parenterally into mice and the induced tumours were almost always solitary or multiple adenomas of the lungs arising within 7–11 months *Mori et al* (1960) found an accurate correlation between the dose of isoniazid and the incidence or the average number of induced pulmonary tumours Their experiments included the testing of a few substances related to isoniazid, and they pointed out the presence of carbamyl groups in all these carcinogenic compounds as in urethane The negative result of *Viallier & Casanova* (1960) may be due to resistence of the Swiss strain mice The weak response to isoniazid in the next test may be connected with the inability of the compound to induce local tumours in mammals or perhaps with the fact that isoniazid is a water soluble compound

The present results with diethylnitrosamine and N nitroso N methylurethane (Table 5) showed that both were active in the next This effect of diethylnitrosamine did not conform with the theory generally accepted at the time of our experiments, that nitrosamines have to be metabolized in the liver in order to become carcinogenic (*Druckrey et al* 1961a b) The metabolic products seem to be the corresponding diazoalkans (*Hultin et al* 1960, *Emmelot et al* 1961, *Druckrey et al* 1961a, b)as in the case of N nitroso-N methyl urethane, but the latter compound releases carcinogenic diazomethane when in contact with any tissue and so it acts as a local carcinogen (*Druckrey et al* 1961b, *Schoental* 1961) This theory has been supported by the predominant induction of liver tumours in rats after peroral application of nitrosamines (*Magee & Barnes* 1956 *Schmahl & Preussmann* 1959, *Schmahl et al* 1960, *Druckrey et al* 1961a *Thomas* 1961), diethylnitrosamine being more effective than dimethylnitrosamine (*Schmahl et al* 1960, *Thomas* 1961, *Argus* 1961) In contrast, feeding of rats with N nitroso-N methyl urethane produces squamous carcinomas of the forestomach and oesophagus (*Schoental* 1960, *Druckrey et al* 1961b, *Schoental & Magee* 1962), while repeated intravenous injections are followed by the appearance of pulmonary carcinomas (*Druckrey & Preussmann* 1962c) N nitroso N methyl urethane is considered a potent carcinogen

A regular occurrence of tumours at other sites than the liver after oral administration of nitrosamines necessitates, however, a modification of the original hypothesis on the mechanism of action Tumours of the kidneys and/or lungs have been observed by many authors (*Magee & Barnes* 1959 and 1962, *Zak et al* 1960, *Argus* 1961, *Argus & Hoch Ligeti* 1961 *Dontenwill et al* 1962), and *Thomas* (1961) saw papillomas and squamous carcinomas of the oesophagus in about one-third of his rats fed diethylnitrosamine in small, daily doses Similar results were obtained in rats with dibutylnitrosamine, these animals developing squamous carcinomas of the oesophagus and the bladder besides hepatic tumours (*Druckrey et al* 1962a) In golden hamsters the organs which were found sensitive to diethylnitrosamine carcinogenesis using any type of application were trachea and the lungs (*Don-*





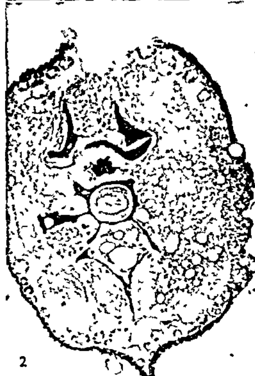


Fig 1

Epithelial hyperplasia and downgrowth on 12th day after injection of N nitroso N methyl urethane (1 per cent) + (40 ×)

Fig 2

Hyperplasia of epidermis and infiltrative epithelial downgrowth on 12th day after injection of diethylnitrosamine (0 5 per cent) + (40 ×)

Fig 3

Positive reaction in Danish newt (Triton vulgaris) on 18th day after injection of benzo(a)pyrene (0 5 per cent Epithelial hyperplasia and downgrowth is seen to the right (10 ×)

ally or parenterally into mice and the induced tumours were almost always solitary or multiple adenomas of the lungs arising within 7-11 months. *Mori et al.* (1960) found an accurate correlation between the dose of isoniazid and the incidence or the average number of induced pulmonary tumours. Their experiments included the testing of a few substances related to isoniazid and they pointed out the presence of carbamyl groups in all these carcinogenic compounds as in urethane. The negative result of *Viallier & Casanova* (1960) may be due to resistance of the Swiss strain mice. The weak response to isoniazid in the newt test may be connected with the inability of the compound to induce local tumours in mammals or perhaps with the fact that isoniazid is a water soluble compound.

The present results with diethylnitrosamine and N-nitroso-N-methyl urethane (Table 5) showed that both were active in the newt. This effect of diethylnitrosamine did not conform with the theory generally accepted at the time of our experiments that nitrosamines have to be metabolized in the liver in order to become carcinogenic (*Druckrey et al.* 1961a, b). The metabolic products seem to be the corresponding diazoalkans (*Hultin et al.* 1960; *Emmelot et al.* 1961; *Druckrey et al.* 1961a, b) as in the case of N-nitroso-N-methyl urethane, but the latter compound releases carcinogenic diazomethane when in contact with any tissue and so it acts as a "local" carcinogen (*Druckrey et al.* 1961b; *Schoental* 1961). This theory has been supported by the predominant induction of liver tumours in rats after peroral application of nitrosamines (*Magee & Barnes* 1956; *Schmahl & Preussmann* 1959; *Schmahl et al.* 1960; *Druckrey et al.* 1961a; *Thomas* 1961), diethylnitrosamine being more effective than dimethylnitrosamine (*Schmahl et al.* 1960; *Thomas* 1961; *Argus* 1961). In contrast, feeding of rats with N-nitroso-N-methyl urethane produces squamous carcinomas of the forestomach and oesophagus (*Schoental* 1960; *Druckrey et al.* 1961b; *Schoental & Magee* 1962) while repeated intravenous injections are followed by the appearance of pulmonary carcinomas (*Druckrey & Preussmann* 1962c). N-nitroso-N-methyl urethane is considered a potent carcinogen.

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tenwill & Mohr 1961, *Dontenwill et al* 1962), in rats subcutaneous injections of diamylnitrosamine induced carcinomas of the lungs and not of the liver (*Druckrey & Preussmann* 1962b) After these various results the theory was abandoned that the action of a specific dealkylating enzyme in the liver was necessary for nitrosamine carcinogenesis, and hence an α-oxidation of the alkyl radical was considered sufficient to start the conversion into diazoalkans, this oxidation being possible in other organs than the liver (*Druckrey & Preussmann* 1962b) Furthermore, *Argus et al* (1961) found diethylnitrosamine and dimethylnitrosamine to be potent protein denaturants and considered it possible, because of the high electron density of the nitroso group, that these compounds might have a direct effect on cells (*Argus & Hoch Ligeti* 1961) *These reviewed considerations conform with the results* in newts

Experiment no 61 (Table 5) illustrates the inactivity of diethyl nitrosamine in watery solution in contrast to the effect in oily solution The explanation may be a rapid resorption of the carcinogen, a problem which has been mentioned in an earlier report (*Arffmann* 1963)

The acute toxic effect of nitrosamines on the liver resembles that of other liver carcinogens (*Barnes & Magee* 1954, *Thomas* 1961) In the newts necrotic lesions of the liver were not observed after diethylnitrosamine, but the dose was small and the application parenteral Feeding *with high doses of N-nitroso-N methyl urethane was followed by acute* destructive lesions of the stomach in rats (*Druckrey et al* 1961b, *Schoental & Magee* 1962), this local toxic effect being comparable with the necrotic lesions of the injection area seen in some newts, when a 10 per cent solution was applied

Triton vulgaris is considerably smaller than Triton cristatus Owing to the viscosity of the oily solutions, finer needles are inapplicable, and the injection therefore more difficult to perform, so that this strain is less suited for routine experiments

CONCLUSION AND SUMMARY

The specificity of the newt test when applied to polycyclic hydrocarbons has been confirmed in supplementary experiments showing complete inactivity of anthracene and triphenylene and weak reaction to 3′,6-dimethyl benz[a]anthracene as compared to dibenz[a h]anthracene in the same low concentrations Quantitative experiments with benz[a]anthracene and chrysene support the conclusion that the degree of response parallels the concentration of the test solution

The negative reaction to 1,2,4-trimethylphenanthrene persisted after raising *the concentration* to 1 per cent

Arsenic trioxide dissolved in water or suspended in peanut oil was negative in the newt test Isonicotinic acid hydrazide in oily suspension induced a weak reaction

Two alkylating agents, diethylnitrosamine and N nitroso-N-methyl-urethane, had a moderate effect in oily solution, while diethylnitrosamine was inactive in water

The last-named four carcinogens represent three, or, at least, two groups of quite different chemical structures, also differing essentially from polycyclic hydrocarbons Yet the results in newts are in reasonable accordance with results in mammals and strongly suggest that the positive conclusion as to the specificity of the newt test may be extended to chemical compounds other than the *polycyclic hydrocarbons*, at least if given in oil

Danish newts (Triton vulgaris) showed the same type of reaction to 3 methylcholanthrene, benzo[a]pyrene and chrysene as did Triton cristatus, but were found less convenient because of their small size

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FROM THE UNIVERSITY DEPARTMENTS OF PATHOLOGY AND OF GYNAECOLOGY ALLMÄNNA SJUKHUSET MALMÖ SWEDEN

EFFECT OF HORMONES ON INCIDENCE OF UTERINE EOSINOPHILIA IN RATS

By

I BJERSING[1] and N E BORGLIN

Received 10 xi 1963

In a previous investigation (2) marked eosinophilia an eosinophilic myometritis, was found in the human myometrium in 15 operated cases Recently *Divack & Janovski* (7) reported moderate to severe degrees of tissue eosinophilia in various female genital organs The cause of this abnormal increase in the number of eosinophilic leukocytes is obscure We found even minor operations on the uterus such as curettage biopsy from the portio or conization of the cervix uteri to be promptly followed by slight eosinophilia This eosinophilia gradually abated but was still demonstrable about 3 weeks after the operation The possibility of the eosinophilia being ascribable to some postoperative endocrine derangement was considered

In investigations on rats (8 18) the number of eosinophilic leukocytes in the uterus was found to vary with the phase of the ovarian cycle In oestrus *i e* when the oestrogenic hormone level is high the number of eosinophilic granulocytes was increased particularly in the mucosa while the number of eosinophilic leukocytes was very low in the immature animal as well as during phases dominated by progesterone *e g* during pregnancy In their study of the effect of oestriol on the hypophysis in spayed rats *Borglin & Bjersing* (3) found marked uterine eosinophilia when large doses of this hormone were given

It is however also known that the adrenocortical steroids have a strong effect on the number of eosinophilic leukocytes in the blood An increased adrenocortical activity due to stress may therefore possibly cause a transient increase in the number of eosinophilic leucocytes in various tissues

The present investigation has the purpose of assessing the effect of different hormones on the number of eosinophilic granulocytes in the uterus of the spayed mature rat

MATERIAL AND METHODS

Female rats weighing 150-200 g were o pl orect mized and ab ut 3 weeks after the operation they were given different h rmones subcutane usly The injections were

[1] Present address Institute of Pathology University of Umeå Umeå Sweden

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The response of the uterine wall to the hormone, *i e*, distension and thinning, oedematous thickening etc has less influence on the semiquantitative method we used for counting the number of cells than on the calculation of the total number of eosinophilic leucocytes When the absolute number of eosinophilic leucocytes is used the uneven distribution must be compensated for by counting the cells within large areas and in a large number of sections, if the accuracy of the results is to be acceptable In view of the large number of animals studied and since the investigation was carried out mainly for purposes of orientation the semiquantitative method which is much less tedious, was decided upon

RESULTS

In intact rats the number of the eosinophilic leucocytes varied with the phases of the sexual cycle The number was larger during pro-oestrus and oestrus than met-oestrus and di oestrus These observations thus confirmed the findings of *Rytomaa* (18) Spaying was followed by the disappearance of all *eosinophilic leucocytes* (*Table 1*)

TABLE 1

Uterine Eosinophilia in Untreated Spayed and Unspayed Rats

Spayed	Unspayed	Number of animals	Cyclic phase	Uterine eosinophilia*	Injected with solvent
	+	5	Di oestrus	I	—
	+	1	Pro oestrus	II	—
	+	3	Oestrus	II	—
	+	1	Met oestrus	I	—
+		2	—	0	—
+		5	—	0	+

* See text

TABLE 2

Uterine Eosinophilia in Spayed Rats Injected with Progesterone Hydrocortisone Corticotropin or Testosterone

Injection of	Total dose	Number of animals	Uterine eosinophilia
Progesterone	5 mg	4	0
Progesterone	12 5 mg	4	0
Corticotropin	30 U	4	0
Hydrocortisone	1 mg	4	0
Hydrocortisone	5 mg	4	0
Hydrocortisone	25 mg	2	0
Testosterone	1 mg	4	0
Testosterone	5 mg	4	0
Testosterone	20 mg	2	0

Injection of large doses of progesterone had no effect on the number of eosinophilic leucocytes in the uterus in the spayed rats Even administration of total doses of *progesterone* of 5 mg and 12 5 mg produced at most very few eosinophilic leucocytes in the uterus and never more than what was found in spayed, untreated animals Neither did administration of testosterone, ACTH or hydrocortisone increase the number

given every other day for 20 days The amount of solution injected at a time was 0 05 ml so that each animal received all together 0 5 ml of solution

The following hormones and solvents were given

Oestrone (pure) dissolved in 9 parts of polyethylene glycol + 1 part of distilled water
Oestriol (pure) dissolved in the same solvent
Oestradiol monobenzoate (Dimenformon®) dissolved in olive oil
Diethylstilboestrol dissolved in peanut oil
Testosterone propionate (Neohombreol®) dissolved in peanut oil
Progesterone (Progestin®) dissolved in peanut oil
Hydrocortisone acetate (Hydrocortal®) dissolved in distilled water
ACTH (Corticotropin A, Acton prolongatum®) dissolved in distilled water

A series of spayed control animals received injections in the same way but only of the solvent polyethylene glycol (9 parts) + distilled water (1 part) A small number of agematched intact rats were also studied In some of these intact animals the effect of testosterone and hydrocortisone was investigated In these groups of intact animals the cyclic phase was determined by vaginal smear which was taken the day before and the day on which the animals were killed These vaginal smears were judged in accordance with criteria given by *Long & Evans* (15) and by *Genell* (10)

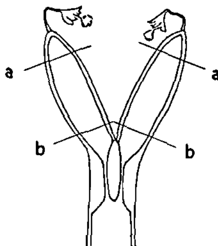


Fig 1
Schematic drawing of rat uterus showing sites from which sections were taken

The animals were anaesthetized with ether and the arteries in the neck were divided The uterus was dissected free and weighed immediately after which it was fixed in 10 per cent formalin for 1 day From each uterine horn pieces of tissue were excised at the sites given in Fig 1 The excised pieces of uterus were embedded in paraffin sectioned (4 μ) and stained with haematoxylin eosin

The number of the eosinophilic leucocytes was judged in the entire section of the uterine horn according to a semiquantitative method with the following grades

0 = at most a few scattered eosinophilic leucocytes per section
I = eosinophilic leucocytes scattered across the whole section
II = moderate number of the eosinophilic leucocytes per section
III = numerous eosinophilic leucocytes per section

No difference in the number of leucocytes was found with the level of the section of the uterine horn or between the left and right horns of the same animal When eosinophilic leucocytes were present they were distributed fairly evenly in the endometrium but unevenly in the myometrium with groups of such cells mainly between muscle bundles and along blood vessels In the endometrium the number of eosinophilic leucocytes was always higher than in the myometrium

The response of the uterine wall to the hormone, i.e., distension and thinning, oedematous thickening, etc., has less influence on the semiquantitative method we used for counting the number of cells than on the calculation of the total number of eosinophilic leucocytes. When the absolute number of eosinophilic leucocytes is used the uneven distribution must be compensated for by counting the cells within large areas and in a large number of sections, if the accuracy of the results is to be acceptable. In view of the large number of animals studied and since the investigation was carried out mainly for purposes of orientation the semiquantitative method, which is much less tedious, was decided upon.

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Hydrocortisone	5 mg	4	0
Hydrocortisone	25 mg	2	0
Testosterone	1 mg	4	0
Testosterone	5 mg	4	0
Testosterone	20 mg	2	0

Injection of large doses of progesterone had no effect on the number of eosinophilic leucocytes in the uterus in the spayed rats. Even administration of total doses of progesterone of 5 mg and 12.5 mg produced at most very few eosinophilic leucocytes in the uterus and never more than what was found in spayed, untreated animals. Neither did administration of testosterone, ACTH or hydrocortisone increase the number

of eosinophilic granulocytes Testosterone was given in doses of 1–20 mg and ACTH was given to 4 animals, to each in a total dose of 30 U ("subcutaneous units") The results are given in Table 2

TABLE 3
Uterine Eosinophilia in Unspayed Rats Injected with Hydrocortisone or with Testosterone

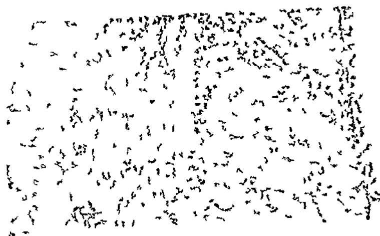
Injection of	Total dose	Number of animals	Uterine eosinophilia	Cyclic phase
Testosterone	1 mg	3	0	Di oestrus
Testosterone	5 mg	3	0	Di oestrus
Hydrocortisone	1 mg	1	I II	Di oestrus
Hydrocortisone	1 mg	1	II	Met oestrus
Hydrocortisone	1 mg	1	II	Oestrus
Hydrocortisone	5 mg	2	I	Di oestrus
Hydrocortisone	5 mg	2	II	Oestrus

TABLE 4
Uterine Eosinophilia in Spayed Rats Injected with Oestradiol Oestrone Oestriol or Diethylstilboestrol

Injection of	Total dose mg	Number of animals	Uterine eosinophilia
Oestradiol	0 001	4	0
Oestradiol	0 005	4	I–II
Oestradiol	0 02	3	II–III
Oestradiol	0 1	3	III
Oestrone	0 005	4	0
Oestrone	0 02	3	I
Oestrone	0 1	3	I II
Oestrone	0 5	3	II–III
Oestriol	1	3	I
Oestriol	5	2	II
Diethylstilboestrol	0 001	4	0
Diethylstilboestrol	0 005	4	I
Diethylstilboestrol	0 02	3	I
Diethylstilboestrol	*0 1*	*3*	*I*
Diethylstilboestrol	0 5	3	II
Diethylstilboestrol	2 5	4	III

The effect of hydrocortisone was also studied in intact rats As in untreated animals, an increase in the number of the eosinophilic leucocytes was found mainly during oestrus The results are given in Table 3, which also gives the effect of testosterone on the intact animals Testosterone inhibited the normal ovarian activity the vaginal smears resembled that found in di-oestrus and the number of eosinophilic leucocytes in the uterus was not larger than in untreated spayed animals

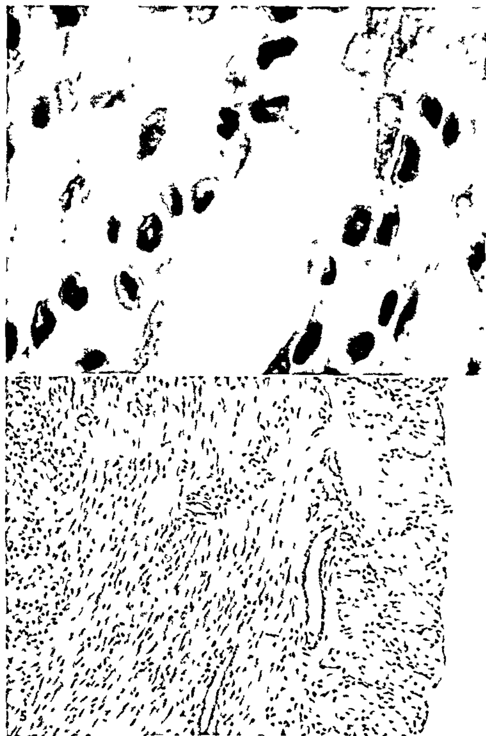
The response to oestrogenic hormones was altogether different We used oestradiol in total doses of 0 001–0 1 mg, oestrone in doses of 0 005–0,5 mg, oestriol in doses of 1–5 mg and diethylstilboestrol in total



F gs 9 an l 3

F 9 Pa t f cr ect n f one horn (b fn s) a ed rat trea ed w th 0 5 mg f e h l t lt e tr l F n phil a f gra le II C oups of e noph ls ar un l es el fn m metr um an l fa l abundant equall d tr b ted cells in mu c a

F g 3 Enlargement of part f F g 2 Eos nophil a of grade II Mult l bular and frequentl annular nucle in the eo n ph l cell



Figs 4 and 5

Fig 4 Enlargement of part of Fig 3 Eosinophilia of grade II Note granules in cytoplasma of eosinophil leucocytes

Fig 5 Unspayed rat during oestrus Eosinophilia of grade II Cf Figs 2 4 Roughly same number and distribution of eosinophil leucocytes

doses of 0 001–2 5 mg per animal All these oestrogenic substances gave rise to eosinophilia varying in severity with the size of the dose given Figs 2–4 show the effect of 0 5 mg of diethylstilboestrol (total dose) This dose produced eosinophilia corresponding to grade II of our method or roughly the same picture as that occurring during oestrus in an intact, untreated animal (Fig 5) Of the oestrogenic substances studied oestradiol appeared to be the most effective Even such a small total dose as 0 005 mg was sufficient to produce marked eosinophilia The smaller dose, 0 001 mg, on the other hand, produced no effect with certainty Oestrone appeared to be only about 1/4 to 1/20 as effective as oestradiol The effect of oestriol was weaker than that of these two others The smallest dose to produce an increase in the number of eosinophilic granulocytes with certainty was about 1 mg Diethylstilboestrol appeared to be about as effective as oestrone (Table 4)

DISCUSSION

The investigation showed that of the hormones tried, only oestrogens increased the number of eosinophilic leucocytes in the uterus in the spayed rat None of the other substances, progesterone, testosterone, hydrocortisone and corticotropin, produced any effect, not even when used in very large doses *The effect of oestrogen is thus specific* This increase in the number of eosinophilic leucocytes in the uterus can be produced by oestrogen in very small doses Oestradiol benzoate even in such a small total dose as 0 005 mg (10 injections of each 0 5 microg

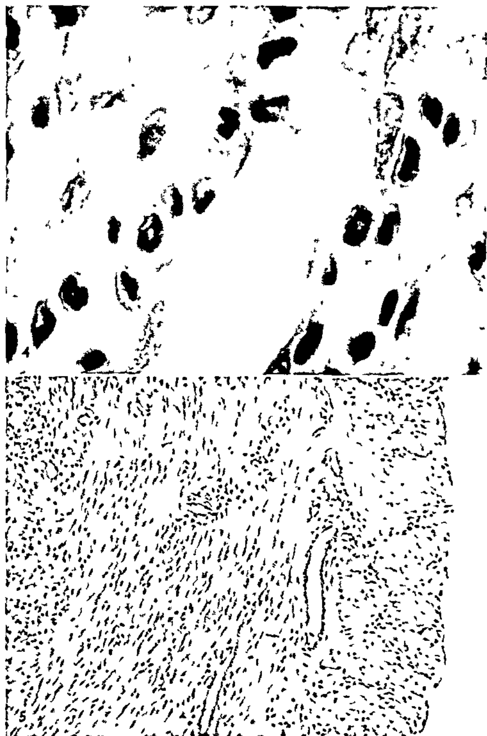
... by the conventional biological oestrogen determination method, *i e*, the vaginal cornification test according to Allen Doisy

It is widely believed that eosinophilic leucocytes are formed in the bone marrow only, whence they enter the blood circulation where they persist for a short time Some authors incline to the view that these cells can also be formed in organs other than the bone marrow, and division of these cells into haematogenic and histiogenic types has been proposed (18)

The increase in the number of uterine eosinophilic leucocytes during physiological or induced oestrus may thus be d...

... (16, 17) who assumed that these cells stem from connective tissue cells and by *Gansler* (9), who claimed to have observed in electron-microscopic studies that these eosinophilic leucocytes derive from smooth muscle cells

The assumption that the increased number of the eosinophilic granulocytes in the uterus is due to an increased inflow of such cells from the blood, is supported by the fact that the number of eosinophilic cells



Figs 4 and 5

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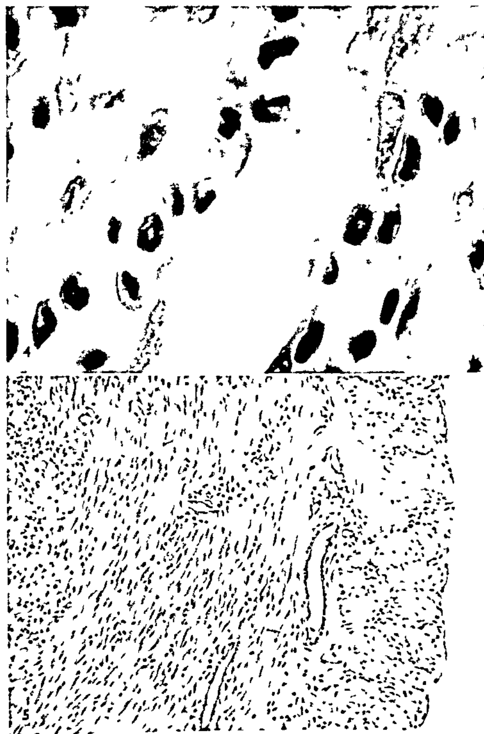
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Fig 5 Unspayed rat during oestrus Eosinophilia of grade II Cf Figs 2 4 Roughly same number and distribution of eosinophil leucocytes

is demonstrable already after such a small total dose as of 0 005 mg of this steroid distributed among 10 injections over 20 days Oestriol has the weakest effect

The results obtained are discussed against the background of the increase in the number of eosinophilic leucocytes after minor operations in homo as demonstrated in a previous investigation

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18 *Rytor*

19 *Shele*

in the blood is low at the time of greatest oestrogen production (4, 6) The origin of these eosinophilic leucocytes is, however, not known with certainty

The physiological rôle of eosinophilic leucocytes is not known either In a recent investigation (1) eosinophilic leucocytes were found to have an antihistamine action (by virtue of their peroxidase activity?) The *eosinophilia induced in the uterus by oestrogens is difficult to explain* Oestrogens have a strong dilatory effect on the vessels in the uterus and accelerate the growth of the uterus, both the endometrium and the myometrium It sounds most likely that the occurrence of the eosinophilic leucocytes in the uterus has something to do with growth

Histamine has a positive chemotaxic effect on eosinophilic leucocytes (1) and a high rate of formation of histamine has been shown in several rapidly growing tissues (12) Mast cells contain and probably also synthesize histamine (14) Induction of the growth of the uterus and vagina in spayed animals by exogenous oestrogen reduces the number of mast cells in the uterus and the vagina (11) A marked eosinophilic infiltration and a complete disappearance of mast cells has been found in induction of decidua cell reaction and in the process of ovum implantation in rats, a reaction which is considered to be associated with histamine release (19) It may be assumed that the increase in the number of eosinophilic leucocytes demonstrated in the present investigation is due to the release or formation of histamine

The increased number of eosinophilic leucocytes after minor operations on the uterus in homo observed in the previous investigation (2) may also be ascribed to liberation of histamine either as a consequence of tissue injury or increased production of oestrogen due to stimulation of the ovaries or adrenals It is known that curettage during the menopause can induce proliferation of the endometrium (5) It has also been shown (13) that vaginal epithelium transplanted to the adrenals in spayed rats shows proliferation after stress, which suggests that stress is capable of stimulating the production of oestrogens in the adrenals

The theories set forth above would thus be able to explain the eosinophilia in the uterus of laboratory animals treated with oestrogens and the eosinophilia demonstrated in homo after minor operations on the uterus

SUMMARY

The effect of hormones on the occurrence of the eosinophilic leucocytes in the uterus was studied in spayed rats Only oestrogens caused an increase *in the number of eosinophilic leukocytes, all of the other* hormones studied (testosterone, progesterone, hydrocortisone and corticotropin) had no effect

Of the oestrogenic hormones, oestradiol which has the strongest physiological effect appeared to be most effective Uterine eosinophilia

with this type of mosaicism and with congenital analgesia were recently reported (*Becak et al* 1963) Structural aberrations in this group of chromosomes have been described in other clinical entities and recently reviewed by *Hamerton* (1962)

The present report concerns the clinical and post mortem findings of a case with trisomy in the group 13 15 Special emphasis will be given to cystic changes of the kidneys with evidence of superimposed infection

CASE REPORT

Clin cal Find ngs

The patient a boy born June 26 1962 and who died October 9 1962 was born at term The parents were unrelated and there were am ng the relatives no apparent malformations inheritable disorders or c nditions kn wn to be connected with chromosomal abnormalities The paternal age was 28 years and the maternal age 21 years at
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anteri r outl ne of the right ventricle was somewhat bulging towards the sternum suggesting a moderate hypertrophy The vascularity of the lungs was increased suggesting a left to-right shunt but the findings at phys cal examination of the heart were not indicative of any distinctive heart lesi n There were no clinical signs of *infection* At the age of three and a half *months* the patient suddenly developed signs of severe *cardiac* decompensation With n 12 *hours the* body weight increased 300 g and liver enlargement peripheral

FROM THE DIVISION OF PAEDIATRIC PATHOLOGY AT THE DEPARTMENT OF PATHOLOGY AND FROM THE DEPARTMENTS OF PAEDITRICS AND ENDOCRINOLOGY AND METABOLISM, KAROLINSKA SJUKHUSET, STOCKHOLM

TRISOMY 13–15 [1]

Report of a Case with Clinical, Cytogenetic and Pathologic Findings

By

C. Blanck, Birgitta Jalling, J. Lindsten and
P. Zetterqvist

Received 14. vi. 63

Autosomal trisomy in the group 13–15 ("D_1 trisomy") in man was first reported by *Patau et al.* (1960). At least 15 cases with this chromosome abnormality have been described in detail in the literature (*Patau et al.* 1960, *Atkins & Rosenthal* 1961, *Lubs et al.* 1961, *Therman et al.* 1961, *Conen et al.* 1962, *Koenig et al.* 1962, *Northcutt* 1962, *Rosenfield et al.* 1962, *Townes et al.* 1962, *Miller et al.* 1963, *Smith et al.* 1963). Since the patients have demonstrated a rather specific clinical picture, on the basis of which they were selected for chromosome studies, it seems likely that the trisomy can be referred to the same long acrocentric chromosome. The most common combination of malformations has been: eye defects ranging from anophthalmia or microphthalmia to coloboma of the iris, low set ears, hare lip and cleft palate, and polydactyly. Less specific features have been heart and brain malformations, deafness, epileptic seizures, and an apparently obligate severe mental retardation. A number of additional anomalies have been observed and recently listed by *Koenig et al.* (1962) and *Smith et al.* (1963).

Chromosome aberrations other than pure trisomy have been described in the group 13–15. *Delhanty & Shapiro* 1962) reported a boy with some of the malformations described above and with one unusually long chromosome in the group 13–15. The precise nature of his chromosome abnormality could not be evaluated. Furthermore, *Warkany et al.* (1962) described a mildly mentally retarded boy with some congenital malformations and with a chromosomal mosaic consisting of apparently normal male cells, and cells with 47 chromosomes and one extra chromosome similar to those in the group 13–15. Two further cases

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1 The Denver system for classification of human mitotic chromosomes was used throughout this work.

The expenses for this study were covered by grants from *Expressens prenatal forskningsfond* to Drs. *B. Ivemark* and *J. Lindsten*.

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The present report concerns the clinical and post mortem findings of a case with trisomy in the group 13 15 Special emphasis will be given to cystic changes of the kidneys with evidence of superimposed infection

CASE REPORT

Clinical Findings

The patient a boy born June 26 1962 and who died October 9 1962 was born at term The parents were unrelated and there were among the relatives no apparent malformations inheritable disorders or conditions known to be connected with chromosomal abnormalities The paternal age was 28 years and the maternal age 21 years at the birth of the patient It was the mother s first child and nothing was known to suggest any previous abortions Prior to conception and during the first part of the pregnancy the mother had been working at a children s hospital as a nurses aid occasionally assisting in holding children at roentgenologic examinations *though* always wearing a lead apron It follows that the gonad doses probably have been negligible Otherwise the pregnancy was uneventful and the delivery normal

The birth weight was 2 600 g and body length 50 cm Multiple external malformations were found including an omphalocoele hare lip and cleft palate (Fig 1) The infant was admitted to the department of paediatric surgery and successfully operated upon for the omphalocoele At the same time a Meckel s diverticulum was resected

At the age of four weeks the infant was transferred to the department of paediatric medicine for further investigations His length was then 52 cm his weight 2 700 g and his head circumference 34 cm On the right side there was an apparent anophthalmia and on the *left side* the eye bulb was very small A bilateral hare lip and cleft palate were present The sagittal suture was wide open and remained so On both hands the boy had an extra digit attached by a bridge of soft tissue to the [illegible] nal

[illegible] of the fourth and fifth sacral vertebrae An intravenous pyelogram was normal [illegible] and lungs was normal at this time [illegible] free of incomplete right bundle branch [illegible] and clock wise rotation of the fronta[illegible] right ventricular hypertrophy An el[illegible]s An audiologic examination accord[illegible] definite hearing impairment

The [illegible] was kept in the hospital for the rest of his life He never showed any psychomotor development From the age of two months he had frequent apnoeic spells with cyanosis There were no feeding problems until two months of age but the weight gain was slow *He then started* to aspirate food and had to be fed by tube Because of the aspirations further roentgenologic examinations of the heart and lungs were performed and a definite progress [illegible]



Fig 1
The patient a few hours after birth

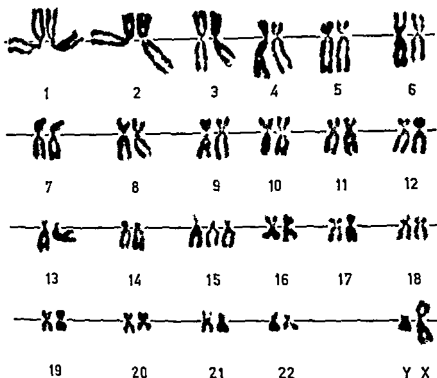


Fig 2

Karyotype from a mitotic leucocyte 47 chromosomes and trisomy in the group 13–15 Acetic orcein stain and phase contrast

oedema persistent cyanosis and dyspnoea were observed Six hours after the first attack of dyspnoea complete atrio ventricular block occurred and finally cardiac arrest During attempts at resuscitation the patient aspirated heavily

Cytogenetic Findings

Chromosome studies were made on cells from cultures of peripheral blood according to a slight modification of the method of *Moorhead et al* (1960) Twenty seven cells were analysed in detail and they all displayed a constant chromosome number of 47 Each cell had seven instead of six chromosomes in the group 13–15, but an otherwise normal male karyotype (Fig 2)

Autopsy Findings

Gross examination—The autopsy was performed 48 hours after death The body length was 56 cm and the weight 4015 g Two normal obliterated umbilical arteries were found There was a bilateral hare lip and cleft palate, and the nasal septum was situated somewhat asymmetrically between the two clefts The external ears were normal apart

from a small skin-clad outgrowth at the helix on the left side On each hand there was a supernumerary finger The umbilical region showed a properly healed scar after the operation of the omphalocoele The right eye was lacking and the left eye bulb was hidden behind a thick fold of conjunctival mucosa and measured only three millimeters in diameter

The *heart* was considered enlarged, and was increased in weight, 31 g after fixation, and presented a dilatation of all the four chambers The walls of both ventricles were of equal thickness, about five millimeters There was a large atrial septal defect of secundum type, measuring about 50 mm in circumference The ostia and valves had a normal configuration, but all the fibrous rings were somewhat wider than normal The venous return was normal, and so were the large arteries The ductus arteriosus was anatomically patent and had an internal diameter of about one millimeter

The *lungs* were normally lobated and weighed 81 g after fixation Moderate atelectasis was present in all lobes The amount of oedema was insignificant The trachea and bronchi contained a considerable amount of white, thick, aspirated material similar to the content of the stomach

In the *abdomen* there was a common mesentery The lower part of the ileum and the oral part of the colon were attached to the posterior abdominal wall by a 15 20 cm long free mesentery There was no vol

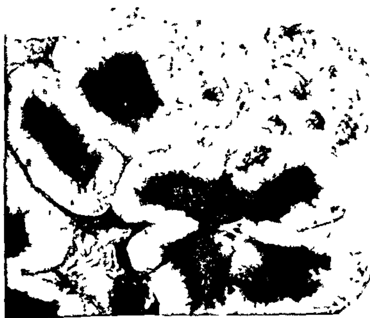


Fig 3

Kidney Note exaggerated lobulation and a cystic area Approximately × 2 5



F g 4

I dn y Low p er v e v of parencl ma n an area h a f larger cysts and some malformed ducts nearby H & F × 30



F g 5

h ln v An a ea w h spa e malf me l ducts and abundant collagenous stroma H & F × 70

vulus. The pancreas appeared normal except for the presence in the tail of a few purplish, less than pea-sized areas of ectopic splenic tissue (Fig. 5). The spleen looked normal except for some deep furrows on its surface. The liver appeared normal

The *kidneys* weighed together 52 g and were considered slightly enlarged. The outer surface was smooth but the lobation was exaggerated (Fig 3). On the cut surface foci of small cysts were found. The cysts measured two millimeters or less in diameter and were located mainly in the medulla (Fig 4). Some cysts were found also in the cortex In the upper poles of both kidneys there were a few yellow, abscess-like areas.

The *urogenital* tract appeared structurally normal and no inflammatory changes of mucous membranes were observed Both testes were found in the abdominal cavity and were of normal size.



Fig 6
Kidney. Flattened excavated papilla H & E × 70

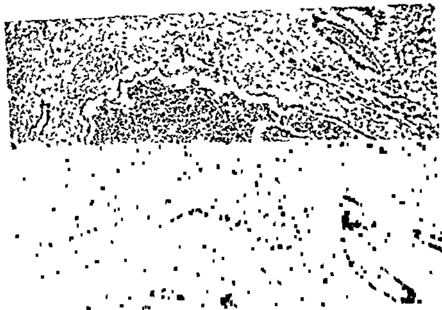


Fig 7

Kidney Acute inflammatory exudation in a cyst and in the adjacent interstitial tissue H & E × 170

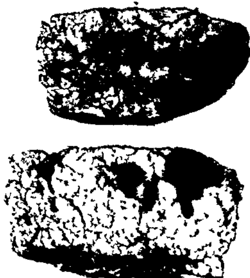


Fig 8

Slices from the tail of the pancreas The dark irregular areas consisted of ectopic splenic tissue × 2 5

The *brain* was grossly normal as were the cerebral vessels

Microscopic examination—In all lobes of the *lungs* considerable inflammatory changes of chronic, non-specific type were found The alveoli contained a moderate amount of a cellular exudate with large macrophages but also with lymphocytes and a few granulocytes There was negligible fibrosis

Areas of cystic changes, predominantly in the medulla, were observed in the *kidneys* The small cysts had a diameter of about 0 1 mm and the largest ones about two millimeters The cysts were lined by epithelium, columnar in the small, and cuboidal or squamous in the large cysts The basement membrane was occasionally thickened and fibrotic The content of some of the cysts was PAS-positive, and other cysts contained a great number of granulocytes (Figs 4, 5, 7) There were also areas of acute interstitial inflammation in the non-cystic parts of the parenchyma Broad strands of dense fibrous tissue were observed between the cysts in the medulla, and there was also a paucity of collecting ducts in these areas The papillae were often flat and occasionally concave (Fig 6) There was no cartilage in the renal tissue

The *pancreas* was normal except for the heterotopic splenic tissue mentioned above Sections from the *myocardium, spleen, liver, adrenals, testes, thymus* and *brain* disclosed no abnormalities The eye was not examined microscopically

Bacteriologic examinations—Moderate growth of *Escherichia coli* was demonstrated in cultures from the kidneys and from one of the lungs

Summary of the autopsy findings—Atrial septal defect of the secundum type with right ventricular hypertrophy and general dilatation of the heart, cystic changes of the kidneys with focal, acute pyelonephritis (Escherichia coli), microphthalmia and anophthalmia polydactyly, bilateral hare lip and cleft palate, slight malformation of one external ear, common mesentery polysplenia omphalocoele and Meckel's diverticulum, chronic non-specific inflammation of the lungs and signs of aspiration

COMMENT

The present case of trisomy in the group 13 15 demonstrated the clinical features usually observed in patients with this chromosome abnormality, *i e*, polydactyly, hare lip and cleft palate, gross malformations of the heart and eyes, and severe mental retardation Though all these features are individually common without suspicion of associated chromosomal abnormality their joint occurrence is highly suggestive of trisomy 13–15 and in fact, have so far provided the basis upon which patients have been selected for cytological examination This was also the case in the present patient The cytogenetical diagnosis, however, was not completed until after the death of the patient and further investigations could not be carried out

Some of the pathological changes observed in the present case are here described for the fist time These are common mesentery, omphalocoele and focal cystic changes of the kidneys Similar pathologic changes of the kidney have been reported in one patient with 48 chromosomes and trisomy in the group 13–15 plus one additional small acrocentric chromosome (*Gustavsson et al* 1962) In this latter case the changes seemed to be more pronounced and also cartilage formation and widespread vascular dysplasia were demonstrated

Furthermore in the present case there were inflammatory changes in the kidneys indicating bacterial infection This finding supports the opinion that *malformations of the renal parenchyma are predisposing* to pyelonephritis (*Fahr* 1938 *Ericsson & Ivemark* 1958 a, b)

SUMMARY

mainly three hitherto undescribed components were also observed common mesentery omphalocoele and cystic changes of the kidneys Inflammation was demonstrated in the kidneys supporting the opinion that malformations of the renal parenchyma predispose to pyelonephritis

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Microscopic examination —In all lobes of the *lungs* considerable inflammatory changes of chronic, non-specific type were found The alveoli contained a moderate amount of a cellular exudate with large macrophages but also with lymphocytes and a few granulocytes There was negligible fibrosis

Areas of cystic changes, predominantly in the medulla, were observed in the *kidneys* The small cysts had a diameter of about 0 1 mm and the largest ones about two millimeters The cysts were lined by epithelium, columnar in the small, and cuboidal or squamous in the large cysts The basement membrane was occasionally thickened and fibrotic The content of some of the cysts was PAS-positive, and other cysts contained a great number of granulocytes (Figs 4, 5, 7) There were also areas of acute interstitial inflammation in the non cystic parts of the parenchyma Broad strands of dense fibrous tissue were observed between the cysts in the medulla, and there was also a paucity of collecting ducts in these areas The papillae were often flat and occasionally concave (Fig 6) There was no cartilage in the renal tissue

The *pancreas* was normal except for the heterotopic splenic tissue mentioned above Sections from the *myocardium, spleen, liver, adrenals, testes, thymus* and *brain* disclosed no abnormalities The eye was not examined microscopically

Bacteriologic examinations —Moderate growth of *Escherichia coli* was demonstrated in cultures from the kidneys and from one of the lungs

Summary of the autopsy findings —Atrial septal defect of the secundum type with right ventricular hypertrophy and general dilatation of the heart, cystic changes of the kidneys with focal, acute pyelonephritis (Escherichia coli), microphthalmia and anophthalmia, polydactyly, bilateral hare lip and cleft palate, slight malformation of one external ear, common mesentery, polysplenia, omphalocoele and Meckel's diverticulum, chronic non-specific inflammation of the lungs and signs of aspiration

COMMENT

The present case of trisomy in the group 13–15 demonstrated the clinical features usually observed in patients with this chromosome abnormality, *i e*, polydactyly, hare lip and cleft palate, gross malformations of the heart and eyes, and severe mental retardation Though all these features are individually common without suspicion of associated chromosomal abnormality their joint occurrence is highly suggestive of trisomy 13–15 and in fact, have so far provided the basis upon which patients have been selected for cytological examination This was also the case in the present patient The cytogenetical diagnosis, however was not completed until after the death of the patient and further investigations could not be carried out

INSTITUTE OF PATHOLOGY, UNIVERSITY OF UMEÅ, UMEÅ, INSTITUTE OF HISTOLOGY, UNIVERSITY OF UPPSALA, UPPSALA, INSTITUTE OF FORENSIC MEDICINE, UNIVERSITY OF LUND, LUND, AND THE KRISTINEBERG ZOOLOGICAL STATION, FISKEBÄCKSKIL, SWEDEN

ON THE AGRANULAR CELLS IN THE PANCREATIC ISLET TISSUE OF THE MARINE TELEOST *COTTUS SCORPIUS*[1]

By

STURE FALKMER, BO HELLMAN, and GERHARD E. VOIGT

Received 14 xi 1963

In addition to the three types of islet cells identified in higher vertebrates (*Hellerström & Hellman* 1960) the pancreatic islets of the bony fish *Cottus scorpius* (the daddy sculpin) contain a considerable proportion of parenchymal cells that invariably lack cytoplasmic granules even ultrastructurally (*Falkmer* 1961, *Falkmer & Olsson* 1962). These agranular cells which are located in the light, peripheral part of the islets, are of particular interest, since ultrastructurally agranular cells have been reported to occur seldom (*Lacy* 1957, *Bencosme & Pease* 1958, *Winborn* 1963) or never (*Björkman et al.* 1963) in higher species.

Since the agranular cells of the sculpin islet tissue thus appeared to represent a fourth cell type of the vertebral islet parenchyma, our previous analyses were extended to these cell... Th
the application of som
cal procedures, as wel
tissue

MATERIAL AND METHODS

Adult daddy sculpin of both sexes were used. Detailed accounts of ...
and the exp ...

... heavy metals were ...
as modified by Vo...
1961) were fixed ...
In addition islets ...
sectioned immedi... ... Heidelberg, Germany). The sections were fixed rapidly in 70 per cent alcohol, placed in distilled water and treated with various reagents for the differentiation of the metal sulphides before physical development (*cf. Timm & Neth* 1959). Trichloroacetic acid

[1] Supported by grants from the Swedish Medical Research Council.

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Fig 1

a) Principal islet of a normal daddy sculpin after application of the sulphide silver method to a cryostat section The central region contains large amounts of heavy metal ions which were concluded to consist essentially of zinc The agranular cells in the peripheral region showed no reaction for heavy metal ions × 35

b) Detail of the same islet (capsule in the lower right corner) The white part represents the non reacting peripheral islet cells × 200

TABLE 1

Results of Some Solubility Tests Applied to Fresh Sections from H_2S Gassed Principal Islets of Cottus scorpius as Compared to Known Reactions of Various Heavy Metal Sulphides

Reagent	Known reactions of various metal sulphides							Islets
	FeS	ZnS	CuS	CdS	PbS	CoS	HgS	
Trichloroacetic acid	—	—	+	+	+	+	+	(+)
Hexacyanoferrous acid	—	—	+	—	+	+	+	(+)
Hexacyanoferric acid	—	—	—	—	—	—	+	—
Potassium cyanide	—	—	—	+	+	+	+	(+)
Hydrogen peroxide	—	—	—		—	—	+	—

The sign + means that the sulphide is not dissolved by the reagent

The sign (+) means that only traces remain of the precipitated sulphide after the solubility test

The sign — means that the precipitated sulphide is completely dissolved by the reagent

firmed by the experiments with intravital chelation of reactable zinc by dithizone and with the magnesium dithizonate method (Fig 2) The agranular cells showed no zinc dithizonate granules in their cytoplasm In the dark field *there* was a whitish luminescent granulation in the central region of the principal islets (Fig 3) The peripheral zone with the agranular cells showed however, no such white cytoplasmic granulation

was used instead of mineral acids and the sections were treated with 20 per cent H_2O_2 for 10 minutes before they were subjected to the sulphide silver procedure The various reagents used in these solubility test *are given* in Table 1 The same solubility tests were carried out on paraffin sections prepared in the way described above In addition the presence of zinc was investigated with the magnesium dithizonate method (*Timm* 1958b) in the cryostat sectioned material and by intravital chelation of the reactive zinc with dithizone (cf *Hellman et al* 1961a) The presence of iron was studied by the Turnbull blue method

The occurrence of luminescent granulation was assessed in darkfield illumination after mounting the fresh cryostat sections in a 50 per cent sorbitol solution (cf *Logothetopoulos & Salter 1960*, *Hellman et al* 1961a) Other sections were fixed in *Bouin's* fluid or 10 per cent calcium acetate formalin (*cf Levine & Glenner* 1958) for a few hours or fixed in formalin vapours for 20 minutes Afterwards the following stains were applied aldehyde fuchsin PAS trichrome a modified *Mallory* procedure (*cf Fallmer* 1961) and the postcoupled benzylidene reaction (*Levine & Glenner* 1958) The A cell staining method of *Lisberg* (1961) was used and the occurrence of metachromasia tested with toluidine blue and Azur A (*cf Manocchio* 1960)

The cell renewal in the principal islets of the sculpin was studied with tritiated thymidine Seven fed, adult sculpin of both sexes weighing between 100 and 140 g, were injected intraperitoneally with 1 mC/kg body weight of a sterile aqueous solution of tritiated thymidine (*Schwarz bioresearch Inc Mount Vernon N Y* lot HTDN 3008, spec act 2 6 C/mM, conc 1 mC/ml) The fish were kept in large aquaria with a constant supply of running sea water and fed on living prawns Four fish were killed 4 hours after the injection and 3 fish after 32 days The principal islets were fixed in *Bouin's* fluid After paraffin embedding 4 µ serial sections were cut and strip film autoradiograms prepared and stained with haematoxylin (*cf Diderholm & Hellman* 1960) In most of these autoradiograms it was possible to localize labelled cells to the central or the peripheral region Since the dark central region occupies roughly about the same part of the total mass of islet tissue as the peripheral region (*Falkmer* 1961) and the various cell types have approximately the same cellular and nuclear size the relative frequency of labelled cells in the two regions could be compared by merely counting all the labelled cells in arbitrarily chosen sections from the two islets To enable more accurate classification of the radioactive cells the film was removed by trypsin digestion (*Baserga & Banks* 1962) or immersion in hot water The remaining paraffin sections were then stained with aldehyde fuchsin for prolonged times (1 or 2 days) The labelled cells in the paraffin sections were located by photomicrographs of the autoradiogram prior to the removal of the film

RESULTS

The sulphide silver procedure showed dense, black granules in the central region of both the paraffin embedded islets fixed in H_2S alcohol and those treated with H_2S gas before they were cut in the microtome cryostat The cells in the peripheral region displayed no reaction for heavy metal ions in their cytoplasm (Fig 1) The solubility tests performed for identifying these precipitated metal sulphides did not give unequivocal results after paraffin embedding Reproducible results were, however, obtained with fresh islets, provided the sections were processed within a few hours The results of the solubility test are given in Table 1 Since the Turnbull blue method gave negative results the H_2S precipitated silver granules were assumed to represent zinc (zinc sulphide) The residual traces of metal after treatment with trichloroacetic acid may have been cobalt or lead provided that certain other metals omitted from Table 1 do not occur in the islets

The findings with the sulphide-silver method were essentially con-

The *Lusberg* method did not yield any useful results as regards the tinctorial features of the sculpin agranular cells These cells were apparently severely vacuolized by the alcoholic fixatives, and the remaining parts of the cytoplasm were only faintly stained No metachromasia was found in the agranular cells with toluidine blue and Azur A The experiments with various fixatives on the cryostat sections and subsequent differential staining procedures gave essentially no further information about the agranular cells beyond that obtained on paraffin sections (*Falkmer* 1961) The agranular cells had only a non specific faint background tinge, and all exhibited a negative reaction with the postcoupled benzylidene method

TABLE 2

Occurrence of Labelled Cells in Different Regions of the two Principal Islets of Cottus scorpius 4 Hours and 32 Days after the Injection of 1 mC/kg Body Weight of Tritiated Thymidine

Time after injection	Overall frequency of labelled cells (per mille)	Number of labelled cells			
		Peripheral region	Central region	Region difficult to classify	Connective tissue
4 hours	2 4	184	111	19	25
32 days	2–4	162	185	14	23

The results of the experiments with tritiated thymidine are given in Table 2 The overall frequency of labelled cells was low Four hours after injection the number of cells with identifiable nuclear radioactivity was higher in the peripheral than in the central islet region (Fig 4) After 32 days the ratio of labelled cells in peripheral to central [illegible] was in many cases impossible to identify the various parenchymal cells In a few experiments, however, it was evident that most of the labelled peripheral cells after the short observation period were among the agranular cells

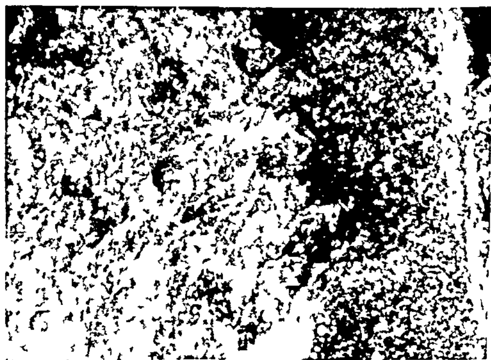
DISCUSSION

In identifying the metal sulphides in the islets with the modified sulphide-silver procedure, reproducible results were obtained with fresh material Experiments with H_2S gassed islets embedded in paraffin were mostly without value, and this was also the case with material kept frozen for more than 24 hours This is most probably due to an ageing process, affecting the sulphides of heavy metals The presence of zinc within the islets was clearly established by the modified sulphide-silver method the dithizonate procedure, as well as by direct chemical determinations (*Falkmer*, unpublished)



I q 2

Pr ncipal islet after applicit on of the magnes m d tl izonate metho l As in Fig 1 there is a j ositive reaction (lark) onl in the islet cells f the ce tral reg on (onnecti e t ssu caj ule n tl e lo r right corner × 180



F g 3

Princ pal islet shov ng a whit sl lt m ne cent dark f el l granulation in the cells of the central region (mid lle and left) l ut no such granulat on in the per phery (right) where the agranular cells occur × 250

The *I usberg* method did not yield any useful results as regards the tinctorial features of the sculpin agranular cells These cells were apparently severely vacuolized by the alcoholic fixatives, and the remaining parts of the cytoplasm were only faintly stained No metachromasia was found in the agranular cells with toluidine blue and Azur A The experiments with various fixatives on the cryostat sections and subsequent differential staining procedures gave essentially no further information about the agranular cells beyond that obtained on paraffin sections (*Falkmer* 1961) The agranular cells had only a non-specific faint background tinge, and all exhibited a negative reaction with the postcoupled benzylidene method

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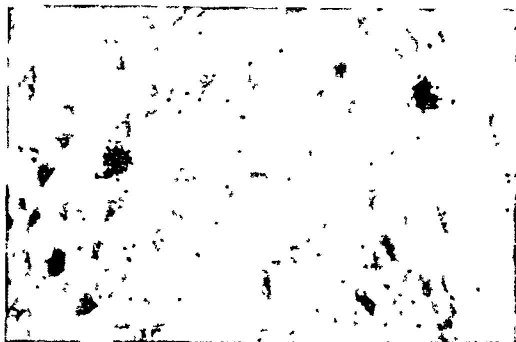


Fig 4

A labelled cell among the agranular cells in the peripheral region (center and left) of a sculpin principal islet in the 'post stained' autoradiogram Near the connective tissue capsule (upper right) there is another labelled cell × 1000

The agranular unclassified cells of the sculpin pancreatic islet tissue are localized to the light peripheral region of the two principal islets and make up the bulk of the parenchyma in the smallest satellite islets, occurring around the principal islets and in the acinar pancreatic tissue of the mesentery They were badly fixed by formalin and all ordinary, alcoholic fixatives, adequate cytoplasmic fixation being only accomplished with strongly protein-precipitating fixatives These cells exhibited only a faint background staining with all the differential staining procedures used *Falkmer* (1961) observed that the agranular cells were unaffected by administration of alloxan, cobaltous chloride, synthalin A, p-aminobenzolsulfonamide isopropylthiodiazole (IPTD), glucose, glucagon, insulin, hydrocortisone, and adrenalin Further, these cells showed no histological changes after asphyxiation and there were no seasonal variations, nor any age or sex differences Ultrastructurally, the agranular cells were found to have a strikingly polymorphous nucleus, fairly dense with evenly distributed chromatin and often one distinct nucleolus (*Falkmer & Olsson* 1962) The cytoplasm was found to be less dense than that of the granular cells and it contained several elongated mitochondria, which sometimes were clumped in juxta-nuclear aggregates Even in their fine structure the agranular cells showed essentially no alterations after alloxan administration

It is widely accepted that incorporation of tritiated thymidine in a cell coincides with the duplication of DNA and reflects synthesis of new

chromosome material The stability of DNA in the cell nucleus except during cell division means that radioactivity derived from tritiated thymidine should be found in cells forming DNA in preparation for division at the time of injection Once labelled cells remain so for long periods depending on the interval before the labelled cell divides again After several mitotic divisions the remaining radioactivity becomes insufficient to produce an autoradiographic image, i e the frequency of labelled cells diminishes The present data about the frequency of labelled islets cells at two observations times may be interpreted in different ways Provided, however, that the time of DNA synthesis in the various types of the parenchymal cells do not differ too much, the higher frequency of labelled cells in the peripheral region at the short observation time suggests a more intensive cell proliferation in this region If so, the ratio of labelled cells between the central and the peripheral regions should diminish with increasing observation times (*Hellman et al* 1961b) In fact the frequency of labelled cells in the peripheral region, seemed to be even lower than in the central region 32 days after injection Another plausible mechanism which may have a bearing on the data of both the short and the long term experiments is that the initially labelled peripheral cells may later appear in the central region This latter mechanism conforms with the supposition that peripheral islet cells to some extent may be progenitors to the granular islet cells These hypothetical peripheral precursor cells would be the *agranular elements* as far as could be deemed from the attempts to identify autoradiographically labelled islet cells after *removing* the strip film

The other features of the agranular islet cells of the daddy sculpin also support the view held by *Bowie* (1924) that the agranular cells in teleostean principal islets might be precursors of the A and B cells The *still* mainly negative histological and histochemical results obtained even after the refined procedures applied, are those, which would be *expected* to be found in incompletely differentiated cell types This view of the *agranular cells* gets further support from the fact, mentioned above, that these cells are mainly localized to the *smallest and probably* still growing islets

SUMMARY

In addition to the three types of islet cells identified in higher vertebrates the pancreatic islet tissue *of the bony fish Cottus scorpius* contains a considerable *proportion* of parenchymal cells that invariably lack cytoplasmic granules even *ultrastructurally* This fourth cell type of the vertebral islet parenchyma is localized to the peripheral region of the principal islets and makes up the bulk of the parenchyma in the small islets in the mesentery and around the two principal islets The agranular cells *exhibit* no characteristic features with several staining

procedures positive for the granular cells and are unaffected by the usual blood sugar altering procedures These observations combined with an autoradiographic study of the incorporation of tritiated thymidine suggest that the agranular cells represent young, immature forms of other islet cells

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FROM UNIVERSITETETS INSTITUTT FOR GENERELL OG EKSPERIMENTELL PATOLOGI RIKSHOSPITALET, OSLO HEAD LEIV KREYBERG, M D, AND UNIVERSITETETS INSTITUTT FOR TROMBOSEFORSKNING, RIKSHOSPITALET, OSLO HEAD PAUL A OWREN, M D

THE HAEMOSTATIC MECHANISM IN PATIENTS WITH HAEMORRHAGIC DISEASES

A Histological Study of Wounds Made for Primary and Secondary Bleeding Time Tests

By

LEIF JØRGENSEN and CHRISTIAN F BORCHGREVINK

Received 5 xi 63

According to *Hugues* (1959a) the haemostatic process in small vessels can be divided into three stages First platelets adhere to the vascular lips Then additional platelets aggregate to form a sufficiently voluminous plug in front of the vascular defect Still, blood may pierce the plug intermittently The third stage occurs within a few minutes and involves solidification of the plug and the bleeding ceases Conventionally, this stage has been associated with fusion of the platelets, a change which is an artefact (*Kjærheim & Hovig* 1962)

There is no doubt that an intact clotting system is of primary importance to the solidification of the plug (*Roskam* 1942, *Hjort, Borchgrevink, Iversen & Stormorken* 1960) *Owren* (1960) and *Borchgrevink & Owren* (1961) showed that the required thrombin is formed on the platelet surface from adsorbed clotting factors

The rôle of the coagulation during the first and second stage is not clear Platelet adhesion and aggregation can be precipitated by thrombin (*Bounameaux* 1955 [illegible] *Schricker* [illegible] 1955, *Witte &* [illegible] 962) The same process ca[illegible] ssive—but not moderate—doses of anticoagulants (*Best, Cowan & MacLean* 1938, *Wright* 1941 *M B Zucker* 1947, *Hugues* 1953, 1959a and b) However, it is unlikely that any clotting product should trigger the initial adhesion of platelets which occurs within one or a few second[illegible] 1959b) [illegible]
pl[illegible]
Bo[illegible]
ch[illegible]

Hugues (1962) and *Hovig* (1962) suggested that the initial adhesion

procedures positive for the granular cells and are unaffected by the usual blood sugar altering procedures These observations combined with an autoradiographic study of the incorporation of tritiated thymidine suggest that the agranular cells represent young, immature forms of other islet cells

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Platelet factor 3 was measured in a thromboplastin generation test system as described by *Biggs & Douglas* (1953)

Proconvertin (Factor VII) was measured by the method of *Aas* (1952), using a substrate plasma from a patient known to have Factor VII deficiency

Prothrombin (Factor II) was measured by the Russel viper venom cephalin m ... with adsorbable se ... method of *Owren* & ...

Thromboplastin time was measured by the method of *Quick* (1935), using human brain thromboplastin

Tourniquet test was performed as described by *Stefanini & Dameshek* (1955 pp 286–288)

Whole blood clotting time was measured by the Lee White method, as described b ...

... n this method is between 3 and 11 minutes

Secondary bleeding time was determined by provoking a renewed bleeding in the same cut 24 hours later (*Borchgrevink & Waaler* 1958) The blood pressure cuff is reapplied and inflated as before The crust of the wound is gently removed with a surgical blade great care being taken neither to cut new vessels nor to cause new tissue damage Normal values for the secondary bleeding time are between 0 and 6 minutes

B ... ndary haemostasis ... the same time as ... minutes after the ...

... utes after the cut was made

The *histological technique* was the same as in previous reports (*Jørgensen & Borchgrevink* 1963 a and b)

RESULTS AND COMMENTS

Unless otherwise stated, there was no important difference between the wounds from patients with the same condition, allowing a joint histological account

Treatment with Heparin and Heparinoid

One normal person (G J) received two intravenous injections of 150 mg of heparin (Heparin "A L", Oslo, Norway, 5 per cent) with 20 hours' interval Fifteen minutes after the last injection the primary bleeding time was normal, the secondary bleeding time slightly increased, and the whole blood clotting time was markedly prolonged (Table 1) The biopsies were performed about 25 minutes after the bleeding was provoked During the excision of the primary wound the bleeding from it started again

One patient with coronary heart disease (A Ø) was treated with the heparinoid dextran sulphate (Dexulate "Glaxo", Greenford, England, 1 000 units per ml) He received two intravenous injections daily for

to the vascular lips takes place on the perivascular connective tissue *Hovig* (1963) has demonstrated that connective tissue fibres cause release of adenosine diphosphate from the platelets *Hellem, Borchgrevink & Ames* (1961) assumed that adenosine diphosphate may also be liberated from the red blood cells *Born* (1962) and *Kaser-Glanzmann & Luscher* (1962) proposed that adenosine diphosphate derived from injured endothelial cells initiates the adhesion, whereas the subsequent aggregation is precipitated by adenosine diphosphate from the platelets formed by the action of small amounts of thrombin generated via tissue thromboplastin

Also other factors are essential to a normal haemostasis

(1) Presence of a sufficient number of adhesive platelets (*Hellem, Borchgrevink & Ames* 1961)
(2) A normal enzymatic content of the platelets, this being defective in thrombasthenia (*Gross, Gerok, Lohr, Vogell, Waller & Theopold* 1960, *Lohr, Waller & Gross* 1961)
(3) A plasmatic factor lacking in von Willebrand's disease (*Nilsson, Blomback & von Francken* 1957)

In previous papers (*Jorgensen & Borchgrevink* 1963a and b) we studied the histology of the normal haemostatic mechanism in standard wounds made for determination of the primary and secondary bleeding times Here we shall report the histology of these wounds in cases with clotting defects, thrombocytopenia, thrombasthenia, and von Willebrand's disease We have two intentions

(1) To get a better insight into pathogenetic mechanisms by correlating our findings with other observations concerning the disease in question
(2) To apply this insight for the elucidation of some of the unsettled questions in the normal haemostasis

MATERIALS AND METHODS

Two persons on anticoagulant therapy and 13 patients with various haemorrhagic diseases form the basis of this study Important clinical data and laboratory findings are summarized in Tables 1 2 and 3 The following diagnostic methods were used

Antihaemophilic A factor (Factor VIII) was measured by the method of *Egeberg* (1961) Dr Egeberg kindly performed the tests

Cephalin time was measured as described by *Egeberg* (1961)

Clot retraction was measured by the method of *Voss* (1958)

Fibrinolysis was measured on standard fibrin plates according to *Astrup & Müllertz* (1952)

Platelet adhesiveness in vitro was measured by the method of *Hellem* (1960)

Platelet adhesiveness (consumption) in vivo was measured in the primary wound by the method of *Borchgrevink* (1960)

Platelet count was carried out in venous blood by the method of *Brecher Schneiderman & Cronkite* (1953) as described by *Borchgrevink* (1960)

7 days, 10–15 000 units in each dose On the day of the biopsies, both the primary and the secondary bleeding times were substantially prolonged, while the whole blood clotting time was only slightly increased (Table 1) The biopsies were performed about 45 minutes after the bleeding was provoked, and while the cuts were still bleeding

In both cases the platelet count, platelet adhesiveness *in vitro* and *in vivo*, as well as clot retraction were normal

Microscopically, the primary plugs were irregular and rich in channels formed by piercing blood streams (Figs 1 and 3) Often plug fragments were floating free within the haematoma (Fig 3) The plugs were partly composed of discrete, loosely aggregated platelets with dense cytoplasm (Fig 1), partly by closely aggregated, apparently fused platelets, and partly by platelets which had assumed the balloon form (*Jorgensen & Borchgrevink* 1963 a and b) The channels contained either no platelets or platelets in the same, or in a less advanced, stage of transformation as the main part of the plug Only the multivesicular plugs had perimetric fibrin membrane and fibrin lining of the channels Only small amounts of fibrin were found in the wounds outside the plugs

In certain respects the plugs of the case under heparin treatment differed from that under treatment with dextran sulphate The former had generally smaller and less irregular plugs, the majority of which was loose textured In the latter case most of the plugs were clearly multivesicular

The secondary plugs were definitely larger than usual in both cases and, in the person under heparin treatment, larger than the primary ones Otherwise they did not differ from the primary plugs, and in both cases the majority had reached the multivesicular stage (Figs 2 and 4) In general, the fibrin strands from the previous day were thinner than usual and the meshes of the net wider More of the fibrin had been removed together with the crust, and more and deeper vessels had rebled Several of the largest vessels in the depth of the wound had bled beneath the fibrin membrane of the walls (Fig 2)

Comments Heparin interferes with the interaction of thrombin with fibrinogen and prevents the conversion of prothrombin in thrombin by inhibition of the formation of prothrombinase ('blood thromboplastin') Dextran sulphate has a similar effect, although qualitative differences do exist (*Hjort & Stormorken* 1957)

The anticoagulant effect was clearly reflected by the deficient fibrin precipitation The drugs had also a profound influence on the platelet plugs, although the platelet aggregation was not clearly retarded Consistently, in experimental thrombosis heparin treatment can prevent fibrin formation without affecting platelet aggregation (*Solandt & Best* 1940, *Poole* 1959) The many traversing channels and the fre[illegible]

[illegible]

ab

TABLE 1

Summary of Clinical Data in 6 Cases with Defective Clotting

Clinical data	Heparin treatment	Dextran sulphate treatment	Haemophilia A		Hypoproconvertinaemia		Normal range
	G J	A O	K S	A T	O Å	J P	
Sex	♂	♂	♂	♂	♂	♂	
Age in years	37	64	29	41	29	18	
Dominating symptom			Joint bleeding	Joint bleeding Haematuria	Epistaxis	Epistaxis	
Primary bleeding time in minutes	8	>30	7	6	5½	9½	3–11
Secondary bleeding time in minutes	9	>30	>30	>30	2½	7	0–6
Platelet count per cu mm (× 1 000)	215	157	304	260	225	199	150–400
No of adhesive platelets *in vitro* per cu mm (× 1 000)	110	74	125	104	66	78	70–140
Per cent adhesive platelets *in vitro*	51	47	41	40	29	39	30–60
No of adhesive (consumed) platelets *in vivo* per cu mm (× 1 000)	82	80	143	91	68	72	45–185
Per cent adhesive (consumed) platelets *in vivo*	38	51	47	35	30	36	25–60
Tourniquet test	Neg	Neg	Neg	Neg	Neg	Neg	
Clot retraction after 3 hours in cm	6.9	6.1	8.0	7.8	8.5	7.5	>6.0
Platelet factor 3	Normal	Normal	Normal	Normal	Normal	Normal	
Whole blood clotting time in minutes	>180	7	45	30	3	3½	2–5
Quick's thromboplastin time in seconds			14.6	15.3	94	86	14–18
PP value in per cent			95	105	9	7	75–125
Cephalin time in seconds			256	224	61.3	64.5	60–70
Prothrombin in per cent			86	94	96	85	75–125
Proconvertin in per cent			106	98	<1	<1	75–125
Antihaemophilic A factor in per cent			<1	<1	94	106	60–150
Fibrinolysis	Neg	Neg	Neg	Neg	Neg	Neg	

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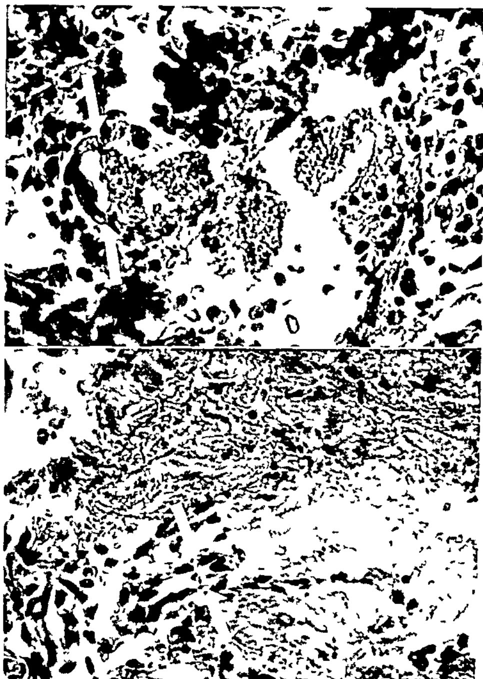
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about [illegible]



Figs 1 2

Fig 1 Person treated with heparin primary bleeding The bleeding restarted during the biopsy A relatively large probably fresh and permeable platelet plug belonging to the capillary vein between the arrows The plug is partly fragmented and consists of loosely aggregated platelets with dense cytoplasm Lendrum's stain × 400

Fig 2 Person treated with heparin secondary bleeding To the right of the arrows a multivesicular plug The bleeding from the capillary vein between the arrows has taken place underneath the old fibrin at the top of the picture Haematoxylin eosin orange fuchsin × 400



Figs 3 4

Fig 3 Pers n treated with dextran sulphate primary bleeding Large irregular plugs and loosened plug fragments with traces of several channels With the exception of the large fragment at the top of the picture the plugs are multivesicular and bordered by fibrin Haematoxylin eosin orange fuchsin × 140

Fig 4 Person treated with dextran sulphate secondary bleeding Large multivesicular plugs crossed by channels Lendrum s stain × 140

plug In their animal experiments both *Apitz* (1942), *M B Zucker* (1947), and *Hugues* (1953) found that moderate doses of heparin gave fragile plugs

It is likely that the fresh look of the primary plugs in the person under heparin treatment indicates that they stem from the restarted bleeding during the biopsy and not from the initial bleeding, which had stopped for several minutes Probably, the majority of the first formed plugs were torn away and just replaced Another observation also shows that during treatment with heparin or heparinoid there is poor permanent fixation of the plugs—and even of fibrin—to the walls of the wound Secondary bleeding had occurred even from deep vessels, often beneath the fibrin membrane of the walls This is a confirmation of the hypothesis of *Roskam* (1942) that heparin makes it more easy to dislodge the platelet plug *Morrison & Doppelt* (1954) measured *in vitro* the firmness with which fibrin is fixed to tissue and found that it increased with increasing amounts of fibrin

The secondary bleeding time in heparinized persons is usually markedly prolonged, most likely due to the defective intrinsic clotting system (*Borchgrevink & Waaler* 1958, *Borchgrevink* 1961 b) In our case the prolongation was only slight, probably because the treatment was somewhat insufficient Nevertheless, the secondary plugs were larger than usual and generally more advanced in their development (*Jorgensen & Borchgrevink* 1963 b) Evidently, they were completed not so near the grossly observed arrest of the bleeding as is usually the case Again, this is a result of poor permanent fixation of plugs and fibrin to the walls of the wound Like in the normal primary bleeding, blood from the deep vessels had probably pierced the plugs and caused continued bleeding for a while, whereas in the normal secondary bleeding only the superficial vessels bleed until the plug is just completed (*Jorgensen & Borchgrevink* 1963 b)

Godal (1962) has shown that platelet factor 4 is more active in neutralizing heparin than dextran sulphate Dextran sulphate depresses the clotting on the platelet surface more than heparin when the anticoagulant effect in plasma is similar (*Borchgrevink* 1961 c) This probably explains the difference in bleeding times and histology in our two cases

Haemophilia A

Two patients with haemophilia A (K S and A T) had normal primary bleeding time, but the secondary bleeding time exceeded 30 minutes (Table 1) They had both less than 1 per cent of antihaemophilic A factor (Factor VIII) The primary wounds were excised about 25 minutes after the cut was made, the secondary wounds after 45 minutes of bleeding

Microscopically, the primary plugs did not differ much from normal primary plugs Most of them were composed of slightly ballooned

platelets (Fig 5), in some of the smaller plugs the platelets had apparently fused The perimetric fibrin membrane was present in the multivesicular plugs, but it was often thin and incomplete The fibrin net outside the plugs was poorly developed

In the secondary wound no real plugs were observed Small aggregates of loosely packed platelets with dense cytoplasm were lying at or near the vascular mouths (Figs 6 and 7) In one of the cases (K S) there were, in addition, larger, irregular, freely floating aggregates of ballooned platelets, surrounded by a perimetric fibrin membrane (Fig 8) Otherwise there were only small amounts of freshly formed fibrin As in the cases under anticoagulant therapy, the fibrin net from the previous day was thinner and its meshes wider than usual Again, more of it had been removed together with the crust and several vessels had rebled underneath the fibrin membrane of the walls pushing the membrane into the gap of the wound (Fig 7)

Comments During the primary bleeding the thrombin necessary to make the plug impermeable is formed through the action of the extrinsic clotting system, triggered by release of tissue thromboplastin This explains the normal primary bleeding time in these patients (*Borchgrevink & Waaler* 1958) However, the plugs and the fibrin net of the primary wounds were somewhat deficient, indicating a slight shortage of thrombin after all This may mean that some of the thrombin developed in the normal primary wound is formed through the *intrinsic* clotting system In accordance with this, *Biggs & Nossel* (1961) observed that activation of the extrinsic clotting system by dilute tissue extract, as encountered in physiological haemostasis, is unable to completely compensate for the lack of the intrinsic clotting system in Factor VIII deficiency

The secondary haemostasis was greatly abnormal, reflecting profound disturbance of the clotting mechanism in these wounds In addition to the *general defect* in the intrinsic clotting system, there is a local insufficiency of the extrinsic clotting system by lack of tissue thromboplastin

The small fresh aggregates in the secondary wounds probably represent an early phase in the development of the platelet plug [illegible] some [illegible] it [illegible] pl[illegible] the aggregates loosen In most of the aggregates neither apparent fusion *nor* balloon formation had occurred, which should mean that they were still reversible This suggests that an incipient clotting is *essential* to the stabilization of the adhesion and aggregation shortly *after* the platelets are arrested, and not only at the stage of permanent solidification of the plug

A continuous formation of easily dislodged and reversible platelet aggregates explains the poor platelet consumption during the haemophilic secondary bleeding (*Borchgrevink* 1961 b) In spite of the severely



prolonged primary bleeding time in the patient treated with dextran sulphate the platelet consumption was normal Probably, the clotting defect in the haemophilic secondary wound is more severe on the platelet surface than in the cases with heparin or heparinoid due to the heparin neutralizing action of platelet factor 4 When patients with clotting defects, including haemophilia A, are treated with oral anticoagulants in order to create severe defects in both clotting systems, even the primary bleeding time becomes prolonged (*Borchgrevink & Owren* 1961, *Owren & Borchgrevink* 1961), still the platelet consumption remains normal The contrast between this and the poor platelet consumption during the haemophilic secondary *bleeding caused Borchgrevink* (1961 b) to postulate an additional local factor in this wound, responsible for the poor platelet adhesiveness This factor may be the inflammatory vascular dilatation (*Jorgensen & Borchgrevink* 1961 b), which even normally causes a reduction in the platelet consumption (*Borchgrevink* 1961 b)

As in the cases under anticoagulant therapy, the platelet plugs and the fibrin from the previous day were poorly fixed to the walls of the wound This is consistent with the well-known recurrence of bleeding in haemophiliacs Most likely, the reduced resistance of the platelet plugs in clotting defects (*Roskam* 1923, *Lewalle, Bounameaux & Roskam* 1959) is primarily a consequence of defective fixation of the first formed plug, and not so much due to friability of the plug *per se*, which is so obvious during actual bleeding in clotting defects

Hypoproconvertinaemia

Two patients (O A and J P) had less than 1 per cent proconvertin (Factor VII), but their primary and secondary bleeding times were normal (Table 1)

Microscopically, the primary plugs were entirely normal (Fig 9) The amount of fibrin outside the plugs was slightly smaller than usual

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Figs 5–8

Fig 5 Haemophilia A (K S) primary bleeding A platelet plug occludes a capillary vein The top of the plug is broken off Although not clearly shown at this magnification the platelets are slightly ballooned Lendrum's stain × 260

Fig 6 Haemophilia A (A T) secondary bleeding Small loose textured platelet aggregate at the mouth of the capillary vein between the arrows Lendrum's stain × 340

Fig 7 Haemophilia A (A T) secondary bleeding Small loose textured platelet aggregate in the homogeneous area of plasma in the centre of the picture The corresponding capillary vein is between the arrows but its lumen is not shown in this slide The bleeding has occurred underneath the old fibrin and pushed it towards the gap of the wound to the left Lendrum's stain × 120

Fig 8 Haemophilia A (K S) secondary bleeding Freely floating platelet aggregates in the multivesicular stage Lendrum's stain × 120

The secondary wound had the same features as those of the cases under anticoagulant therapy, *viz*, irregular plugs, rich in channels freely floating aggregates, and bleeding from deeper vessels than usual, often beneath the fibrin membrane of the walls (Fig 10)

Comments Patients with hypoproconvertinaemia (Factor VII deficiency) have a defective extrinsic clotting system, whereas their intrinsic clotting system is normal The normal primary plugs must indicate that the intact intrinsic clotting system in these patients provides sufficient thrombin for a normal haemostasis This is in agreement with *Waaler* (1957) who found that tissue thromboplastin may trigger the intrinsic clotting system in proconvertin-deficient plasma

The secondary wound in hypoproconvertinaemia showed definite signs of a defective coagulation This defect, however, is milder than that of the haemophilic secondary wound The interpretation of our observation is difficult, but it probably indicates that both clotting systems are necessary for a normal haemostasis in the secondary wound Even in these wounds there is probably a slight, but important release of tissue thromboplastin This may vary somewhat, but it must be to a considerably smaller degree than that in the primary wound, considering the great difference between the primary and secondary wound in haemophilia A

Macroglobulinaemia

Two patients with macroglobulinaemia (A J and O M) had platelet counts at or slightly below the lower normal limit (Table 2) The number af adhesive platelets *in vivo* was definitely reduced, while *in vitro* it was nearly normal In one of the patients (A J) both the primary and secondary bleeding times were markedly prolonged, and in this case the biopsies were taken 45 minutes after the bleeding was provoked In both cases the clotting tests were normal or showed slight deficiencies (Table 2) The erythrocyte sedimentation rate was more than 100 mm per hour, and Sia's test was positive The total protein in serum was 8 5 and 9 7 gm/100 ml respectively, the gamma globulin 5 6 and 5 8 gm/100 ml The diagnosis of macroglobulins was confirmed by ultracentrifugation

Microscopically, both the primary and secondary plugs were multivesicular, bounded by a distinct fibrin membrane, but they were small and inconspicuous (Fig 11) They were crossed by several channels with marked fibrin linings, and loosened plug fragments were occasionally encountered within the haematoma The amount of fibrin was larger than expected and fibrin bands had formed ring figures (Fig 12) Numerous particles of platelet origin adhered to the fibrin net The serum stained as fibrin

Comments *Braunsteiner, Falkner, Neumayer & Pakesch* (1954) suggested that pathological proteins coat the platelets in macroglobulin-



Figs 9–10

Fig 9 [illegible] Normal multivesicular

Fig 10 [illegible] Freely floating aggre- [illegible] The mass at lower left is a retained part of the old crust Between the right pair of arrows the old fibrin membrane of the wall of the wound is loosened and bleeding has taken place underneath Lendrum's stain × 300

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Comments *Braunsteiner, Fallner, Neumayer & Pakesch* (1954) suggested that pathological proteins coat the platelets in macroglobulin



Figs 11 12

Fig 11 Macroglobulinaemia (A J) primary bleeding Small plug belonging to the capillary vein between the arrows nevertheless it is the largest plug observed in these cases Several traces of channels are lined with fibrin Lendrum s stain × 300

Fig 13 Macroglobulinaemia (A J) primary bleeding The wound after 45 minutes of bleeding Fibrin bands have formed ring figures The serum stains as fibrin and appears as homogeneous fragmented areas No plug is visible Haematoxylin-eosin orange fuchsin × 35

TABLE 2

Summary of Clinical Data in Patients with Macroglobulinaemia, Thrombocytopenia and Thrombasthenia

Clinical data	Macroglobulinaemia		Idiopathic thrombocytopenic purpura	Pancytopenia	Thrombasthenia		Normal range
	A J	O M	I I	G J	K H	S B	
Sex	♂	♂	♀	♂	♂	♂	
Age in years	80	71	60	26	18	15	
Dominating symptom	Epistaxis		Purpura	Purpura Intestinal bleeding	Epistaxis Intestinal bleeding	Epistaxis	
Primary bleeding time in minutes	>30	10	26	>30	>30	>30	3–11
Secondary bleeding time in minutes	>30	8			>30	>30	0 6
Platelet count per cu mm (× 1 000)	102	150	24	32	128	188	150 400
No. of adhesive platelets *in vitro* per cu mm (× 1 000)	45	74			2	4	70–140
Per cent adhesive platelets *in vitro*	44	49			1 6	2 1	30 60
No. of adhesive (consumed) platelets *in vivo* per cu mm (× 1 000)	3	21	13	16	4	0	45–185
Per cent adhesive (consumed) platelets *in vivo*	1	14	55	50	3	0	25 60
Tourniquet test	Doubtf	Neg	Pos	Pos	Pos	Pos	
Clot retraction after 3 hours in cm	6 2	7 0	4 0	3 8	2 0	1 5	>6 0
Platelet factor 3	Normal	Normal	Normal	Normal	Normal	Normal	
Whole blood clotting time in minutes	4½	4	4	3½	3	3	2 5
Quick's thromboplastin time in seconds	17 2	16 4	14 9	13 8	14 0	14 4	14–18
PP value *in per cent*	65	78	90	115	100	92	75–125
Cephalin time in seconds	74 1	66 5	61 3	63 0	62 8	64 0	60 70
Prothrombin in per cent	68	75	90	100	90	96	75 125
Proconvertin in per cent	78	82	88	110	102	92	75–125
Antihaemophilic A factor in per cent	82	90	108	95	95	109	60 150
Fibrin lysis	Neg	Neg	Neg	Neg	Neg	Neg	



Figs 13 14

Fig 13 Idiopathic thrombocytopenic purpura primary bleeding The largest plug observed in the cases of thrombocytopenia occluding both the arteriole at lower right and the vein at upper right Several channels traverse the plug Lendrum's stain × 300

Fig 14 Thrombasthenia (K H) primary bleeding A loose collection of unaltered platelets in the bottom of the wound after 6 minutes of bleeding The fine fibrin strands enclosing the platelets are scarcely seen at this magnification Haematoxylin eosin orange fuchsin × 300

aemia *Jurgens* (1956) and *Pachter, Johnson, Neblett & Truant* (1959) showed that they interfere with the release of platelet factor 3 Electron microscopy have revealed defective pseudopod formation (*Braunsteiner, Falkner, Neumayer & Pakesch* 1954)

In accordance with previous findings (*Borchgrevink* 1961 a), the platelet consumption *in vivo* in our cases of macroglobulinaemia was poor, although the adhesiveness *in vitro* was normal The small platelet plugs are readily explained by deficient adhesion and aggregation The reason for this is not clear However, it is not likely that this is due to the poor release of platelet factor 3 because there was no difference between the primary and secondary wounds in the severity of bleeding or in the histological findings It seems more probable that the coating of the platelets by macroglobulins makes them partly non-reacting to the normal triggering mechanisms

The many channels through the plugs and the loosened plug fragments may be consequences of slow aggregation The plugs are not being built up fast enough to reach the volume necessary to withstand the intravascular pressure before they are broken down again However, this histological picture, as well as the poor adhesion and aggregation, may also be compatible with a clotting defect This must then be limited to the platelet surface, considering the normal clotting tests and the rich fibrin net This explanation is supported by the theories that the thrombin necessary to haemostasis is formed on the platelet surface (*Owren* 1960, *Borchgrevink & Owren* 1961), and that macroglobulins may interfere with the clotting process (*Henstell & Kligerman* 1958)

Thrombocytopenia

Two patients had thrombocytopenia, one idiopathic thrombocytopenic purpura (J F), the other pancytopenia (G J) Their platelet counts were 24,000 and 32,000 per cu mm respectively, and the primary bleeding time was prolonged (Table 2) Secondary bleeding time was not measured in these patients Clot retraction was delayed and tourniquet test was positive, while all the other platelet function and clotting tests were normal

Microscopically, both patients had formed several plugs of varying size, most of them small, but some were fairly large (Fig 13) They were crossed by several channels and plug fragments were floating free in the haematoma Otherwise the structure of the plugs was normal and the amount of fibrin was as expected Even here, rings of fibrin bands were encountered, and the fibrin net contained particles of platelet origin as usual

Comments The number of platelets in our cases was considerably reduced, but the percentage of adhesive platelets *in vivo* was unaltered However, more important is the absolute number of adhesive platelets *Hellem, Borchgrevink & Ames* (1961) found that the bleeding time was prolonged when the number of adhesive platelets was below 40,000



Figs 13, 14

Fig 13 Idiopathic thrombocytopenic purpura, primary bleeding. The largest plug observed in the cases of thrombocytopenia, occluding both the arteriole at lower right and the vein at upper right. Several channels traverse the plug. Lendrum's stain. × 300

Fig 14 Thrombasthenia (K. H.), primary bleeding. A loose collection of unaltered platelets in the bottom of the wound after 6 minutes of bleeding. The fine fibrin strands enclosing the platelets are scarcely seen at this magnification. Haematoxylin eosin orange fuchsin. × 300

per cu mm In our cases it was only between one third and a half of this

Our findings in thrombocytopenia resembled those in macroglobulinaemia, but the changes were more moderate This is perhaps not surprising, considering the reduced number of platelets capable of adhering and aggregating in both conditions Even an abnormal protein coating of the platelets may be a common feature, for platelet agglutinins are often found in serum of patients with various forms of thrombocytopenia (*Harrington & Arimura* 1961); in pancytopenia they may be secondary to multiple transfusions With a modified Coombs' test it has been shown that the platelets in idiopathic thrombocytopenic purpura are coated by incomplete agglutinins (*Fluckiger, Hassig & Koller* 1954) Thus, many of the same deliberations as in macroglobulinaemia can be made in thrombocytopenia Even here the signs of delayed solidification may point to a disturbed clotting on the platelet surface

Our findings of platelet plugs in thrombocytopenia is in accordance with *Apitz & Huhn* (1943) in an experimental study on rats, but at variance with all other histological studies on thrombocytopenia, both in man and in animals (*Muller* 1931, *Apitz* 1943, *M B Zucker* 1947, *H D Zucker* 1949, *Monto* 1961) We can offer no explanation for this

Thrombasthenia (Glanzmann's Disease)

Two patients with thrombasthenia (K H and S B) had markedly prolonged primary and secondary bleeding times, reduced number of adhesive platelets both *in vivo* and *in vitro*, positive tourniquet test, and delayed clot retraction (Table 2)

In one of the patients (K H) we obtained biopsies of the primary wound both 6 and 45 minutes after the cuts were made We refrained from biopsy of the secondary wound in this case

Microscopically, the primary wound 6 minutes after the cut contained loose-textured, poorly delimited platelet masses at or near the vascular mouths (Fig 14) The platelets had dense cytoplasm, were discrete and mostly entrapped in a dense fibrin net with delicate strands and irregularly sized meshes (Fig 15) The fibrin contained no particles of platelet origin Individuals or groups of red and white blood cells were mixed with the platelets

In the primary and secondary wound 45 minutes after the start of the bleeding similar collections of platelets were encountered but now they were larger (Fig 16) The fibrin net was remarkably well developed and fibrin rings had formed

Comments Functionally, the platelets in thrombasthenia (Glanzmann's disease) are characterized by poor adhesiveness both *in vivo* (*Borchgrevink* 1961 a) and *in vitro* (*Hellem* 1960) Morphologically the platelets show deficient pseudopod formation and the fibrin net is not oriented towards platelet centres (*Braunsteiner* 1955) Studies by *Gross, Gerok, Lohr, Vogell, Waller & Theopold* (1960), *Lohr, Waller & Gross*

(1961) and *Larrieu, Caen, Lelong & Bernard* (1961) have revealed that the basic defect is one of two metabolic derangements, one leading to insufficient production of adenosine triphosphate, the other to deficient utilization of it. This substance is required for the contraction of the contractile protein, a reaction which underlies the thrombin induced alterations of the platelets (*Bettex Galland & Luscher* 1960, *Grette* 1962).

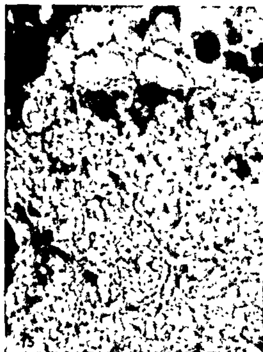
The non reactivity of the thrombasthenic platelets is also evident in our slides. In spite of this a sort of platelet collections has formed, but they were loose textured obviously unable to resist the blood flow. The thrombasthenic platelets are unaffected both by adenosine disphosphate (*Hellem* 1960) the connective tissue factor (*Zucker & Borelli* 1962) and the clotting process (*Braunsteiner* 1955, *Sokal* 1960, *Sharp* 1961). They seem to be kept together by the fibrin net which criss crosses the collections.

In accordance with previous reports we found no clotting defect in our cases. In fact the amount of fibrin in the wounds was even greater than expected. A hyperactive clotting milieu is conceivable due to a freer exchange of clotting factors between the platelets, here lying scattered and the plasma. In normal wounds this cannot be expected to take place to the same degree because many of the platelets are "locked up" in plugs.

TABLE 3

Summary of Clinical Data in Patients with von Willebrand's Disease

Clinical data	K M	C H	A B	Normal range
Sex	♀	♀	♀	
Age in years	40	28	45	
Dominating symptom	Menorrhagia	Menorrhagia Epistaxis	Menorrhagia	
Primary bleeding time in minutes	>30	>30	>30	3 11
Secondary bleeding time in minutes	>30	>30	>30	0-6
Platelet count per cu mm (× 1 000)	332	298	296	150-400
No of adhesive platelets in vitro per cu mm (× 1 000)	128	134	14	70-140
[illegible]	33	45	49	30-60
[illegible]	7	9	3	45 185
[illegible]	2	3	1	25-60
Tourniquet test	Pos	Pos	Pos	
Clot retraction after 3 hours in cm	8 2	7 9	8 4	>6 0
Platelet factor 3	Normal	Normal	Normal	
Whole blood clotting time in minutes	4	4½	3	2 5
Quick's thrombplastin time in seconds	14 9	15 3	15 6	14 16
P-P value in per cent	102	94	82	75 125
Cephalin time in seconds	92 0	85 0	74 0	60-70
Prothrombin in per cent	94	104	80	75-125
Proconvertin in per cent	90	94	103	75-125
Antihaemophilic A factor in per cent	7	10	42	60 150
Fibrinolysis	Neg	Neg	Neg	


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von Willebrand's Disease

Three patients with von Willebrand's disease (K M, G H, and A R) had primary and secondary bleeding times exceeding 30 minutes The platelet adhesiveness *in vivo* was reduced but *in vitro* it was normal (Table 3) The antihaemophilic A factor (Factor VIII) was reduced in all cases but platelet factor 3 was normal

In one of the patients (A R) the biopsy of the primary wound was performed 6 minutes after the cut, in the other two patients the wounds were excised after 45 minutes, while still bleeding Biopsies of the secondary wounds were not carried out

Microscopically, after 6 minutes, there was no platelet plug in front of the bleeding vascular mouth (Fig 17) No platelets adhered to the damaged endothelium instead aggregates of apparently fused platelets adhered to the connective tissue of the walls of the wound between the vessels In certain parts of the wound a normal superficial fibrin membrane covered the walls

After 45 minutes platelet plugs were still not present (Fig 18) However, the gap of the wounds was nearly filled by large platelet aggregates fixed to the walls between the vessels Most of the aggregates were multivesicular and surrounded by a thick and poorly defined fibrin membrane even towards the wall Some of the aggregates had under

longed

VIII)

sson, Blombäck & von Francken (1957) showed that a plasma factor present in Cohn's fraction I 0, not identical with Factor VIII or fibrino-

Figs 15–18

Fig 15 Th ... collection of many ... At the top of the ... sized meshes Len

Fig 16 Thrombasthenia (S B) secondary bleeding A large collection of platelets extends through the entire length of the picture The bleeding vessels are between the arrows at the bottom Within the collection fibrin strands form a dense net The darker nearly homogeneous areas ob... are c...

Fig 17 ... vein ...

Fig 18 ...


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18

this disease suggest that adhesion to connective tissue—and subsequent aggregation—may occur even in the presence of a defective initial adhesion to the endothelium The adhesion to connective tissue and aggregation are probably precipitated by the substance of *Zucker & Borrelli* (1962) and/or more ample amounts of adenosine diphosphate large enough to overcome the relative insensitivity of the platelets in von Willebrand's disease

Our findings in thrombasthenia confirm the importance of a normal platelet metabolism to an effective adhesion and aggregation Vascular dilatation probably reduces the initial adhesion and aggregation (*Borchgrevink* 1961b *Jorgensen & Borchgrevink* 1963b) and this is probably reflected also in this study by the particularly poor platelet adhesion and aggregation in the haemophilic secondary wound

The small loose textured platelet aggregates in the primary wound of the heparinized person and in the haemophilic secondary wound may signify that coagulation is not involved in the early reversible aggregation However we cannot exclude an effect of trace amounts of

the case may be The action of thrombin at this stage may be twofold Thrombin induces contraction of the contractile protein (*Grette* 1962) and thereby release of adenosine diphosphate from the platelets (*Kaser-Glanzmann & Luscher* 1962) This precipitates clumping of additional platelets The other effect of thrombin is probably best illustrated in the haemophilic secondary wound Here the easily dislodged and reversible aggregates indicate that small amounts of thrombin stabilize the adhesion and aggregation shortly after the platelets are arrested

The great importance of the coagulation to the permanent impermeability of the plug is obvious from all cases with clotting defect This does not only imply mutual attraction of the platelets but also a more solid fixation of the plug to the vessel Both the extrinsic and intrinsic clotting systems participate although to a varying degree according as the haemostasis is of the primary or secondary type In the primary wound the clotting systems are largely able to compensate for each other In this respect the intrinsic clotting system is the more efficient one creating completely normal primary plugs in Factor VII deficiency In the secondary wound the lack of tissue thromboplastin and the vascular dilatation are handicaps to the haemostatic mechanism Although the intrinsic clotting system is the most important here the secondary wound in Factor VII deficiency indicates that the extrinsic clotting system participate to a certain but significant degree

Our findings in macroglobulinaemia and thrombocytopenia are ambiguous They may signify that the building up of the plug must take place with more than a minimum of speed If not the plug does not reach the volume necessary to withstand the intravascular pressure before

gen, was able to correct the bleeding time. The platelet adhesiveness *in vitro*, determined with the method of *Hellem* (1960), is normal, but when measured by a method avoiding decalcification (*Salzman* 1963) it is diminished. *Borchgrevink* (1961 a) found reduced platelet consumption *in vivo* and concluded that the prolonged bleeding time might be explained by lack of, or delay in plug formation.

In our cases there were really no platelet plugs, but aggregates adhered to the walls of the wound between the vessels. No platelets adhered to the endothelial lips. This suggests that the initial adhesion to the endothelial cells is disturbed, whereas the adhesion to connective tissue—and the subsequent aggregation—is normal. This has important implications. It means that the mechanism which triggers the platelet adhesion to damaged endothelium is not identical to that inherent with connective tissue. Our findings agree well with *Zucker & Borrelli* (1962) who observed that platelets in von Willebrand's disease reacted normally towards the substance in connective tissue. *Caen & Cousin* (1962) and *Caen* (1963) reported that patients with von Willebrand's disease had raised plasma level of adenosine triphosphate without increase in adenosine diphosphate. *O'Brien* (1962) and *Born* (1962) found that the former substance counteracts the latter in its action on the platelets. *Ødegaard, Skålhegg & Hellem* (1963) were able to show more directly that the platelets in von Willebrand's disease have reduced sensitivity towards small amounts of adenosine diphosphate. Thus, the impaired initial adhesion to endothelium may be due to this reduced sensitivity. If so, it also indicates that adenosine diphosphate is the substance which normally triggers the first platelet adhesion to the damaged endothelium.

DISCUSSION

The histological method of studying defective haemostasis gives information about morphological changes of the plugs and altered relations of the plugs to other structures. This information is missing in most studies on haemostasis and the morphological concept of the process *in vivo* is formed only by inference. However, it may be difficult to interpret the histological observations and, due to the small number of cases in this study, definite conclusions can hardly be drawn. On the other hand, the morphological variations between similar cases were small and, usually, the findings agreed with pathophysiological observations.

On the basis of this correlative study of defective haemostasis certain suggestions can be formed even concerning the normal haemostasis. As to the initial platelet adhesion to the endothelial lips, this seemed to be impaired in von Willebrand's disease, which probably indicates that small amounts of adenosine diphosphate triggers this reaction. On the other hand, the aggragates well fixed to the walls of the wound in

were criss crossed by a rich fibrin net with irregular meshes No plugs were found

In von Willebrand's disease platelet aggregates adhered to the connective tissue between the bleeding vessels No platelets adhered to the endothelial lips

The following conclusions are drawn

From the findings in von Willebrand's disease it is likely that the initial platelet adhesion to the endothelial lips is triggered by small amounts of adenosine diphosphate

The subsequent aggregation may be caused by larger amounts of adenosine diphosphate or the connective tissue but an intact clotting is probably necessary in order to stabilize the adhesion and aggregation early in the plug formation

The great importance of an intact clotting to permanent impermeability is confirmed but it is emphasized that this not only implies a firmer mutual attraction of the platelets but also a more solid fixation of the plug to the vessel

Both the extrinsic and intrinsic clotting system participate in both the primary and secondary haemostasis In the secondary haemostasis the intrinsic clotting system is the most important even in the primary haemostasis a defective intrinsic clotting system is not completely compensated by an intact extrinsic clotting system

The difference in the platelet transformation when a plug is formed and when a fibrin net is produced depends on the local thrombin concentration Within the plug the amount of thrombin is probably small resulting in ballooning of the platelets Within the haematoma the amount of thrombin is larger causing loss of platelet individuality or integrity

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it is broken down again However, the observations may also, particularly in macroglobulinaemia, reflect a local disturbance of the important clotting on the platelet surface

In the cases with clotting defect there were both plugs with apparently fused platelets without fibrin, and multivesicular plugs with fibrin coating of the periphery and channels This support our theory that the ballooning of the platelets represents a later stage in the post haemorrhagic development of the plug (*Jorgensen & Borchgrevink* 1963a and b) Further, the close association between the ballooning and the fibrin formation suggests that the former is also produced by thrombin, probably at the height of its effect Nevertheless, no fibrin is found between the platelets in the plug shortly after bleeding (*Kjærheim & Hovig* 1962) To form fibrin still larger amounts of clotting factors may be necessary We may conceive that within the stagnant milieu on the platelet surfaces within the plug thrombin is rapidly inactivated and the amount of fibrinogen is limited No more plasma enters from outside through the extremely narrow slits between the platelets Only at the periphery and along the channels fresh supplies of clotting factors are available in amounts necessary to precipitate fibrin On the other hand, between the loosely collected thrombasthenic platelets fibrin precipitation can take place throughout the platelet mass

In the haematoma of the normal wounds platelets form small scattered aggregates under the influence of ample thrombin The platelets loose their individuality or integrity and leave particles of fused granulomers as centres in the fibrin net (*Selna & Rosenthal* 1958, *Sol al* 1960) By electron microscopy *Hovig* (1962) found that small amounts of thrombin do not rupture the platelet membranes, while larger amounts do Most likely, this accounts for the difference between the platelet transformation in a plug and in a fibrin net (*Parmeggiani* 1961, *Jorgensen & Borchgrevink* 1963a)

SUMMARY AND CONCLUSIONS

Histological examination of wounds made for the primary and secondary bleeding time tests was carried out in 15 cases of haemorrhagic disorders, including 2 cases under treatment with heparin and dextran sulphate respectively The excision of the wounds was performed from 6 to 45 minutes after the bleeding was provoked

Clotting defects (anticoagulant therapy, haemophilia A, and hypoproconvertinaemia) tended to give small, permeable, friable plugs which were easily dislodged and often delayed in their development The most severe defects were found in the secondary wound of haemophilia A

In macroglobulinaemia and thrombocytopenia similar plugs were observed, in spite of normal clotting tests and a well developed fibrin net outside the plugs

In thrombasthenia loose textured collections of unaltered platelets

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THE UNIVERSITY INSTITUTE FOR EXPERIMENTAL MEDICINE
NORRE ALLE 71 COPENHAGEN DENMARK

RENIN IN NEPHROGENIC RENAL TISSUE DEVOID OF BOTH GRANULAR AND NON GRANULAR EPITHELOID JUXTAGLOMERULAR CELLS

By

JENS BING and JERZY KAZIMIERCZAK

Received 17 vi 63

The high correlation between juxtaglomerular granularity and renal renin content has been taken as a sign that renin is formed in the granulated juxtaglomerular cells (*Goormaghtigh* 1944 *Dunihue & Candon* 1940 *Hartroft* 1957 *Gross* 1960 and *Tobian* 1960) This opinion has been stressed by studies with fluorescent antibodies against a purified but not pure renin preparation (*Hartroft & Edelmann* 1960), and by direct analyses of microdissected renal tissue (*Peart Gordon Cook & Pickering* 1956 1959 1960 and *Bing & Kazimierczak* 1960) In previous studies (*Bing & Kazimierczak* 1962) it was found however, that from well over 50 to well over 90 per cent of the renin is located in the part of the distal tubule which includes the macula densa and that only a smaller fraction is found in extracts of the afferent vessels These results made it likely that renin is formed in the macula densa and that part of it is deposited in the afferent arteriole This conclusion agrees well with the result of studies of *Kaplan & Friedman* (1942) who found renin in the mesonephros and metanephros of the hog foetus at an age when the juxtaglomerular apparatus is undeveloped

This paper contains a reinvestigation of the question does renal tissue devoid of vasa afferentia and thus devoid of both granular and non granular juxtaglomerular cells contain renin? W [illegible]

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This paper contains a reinvestigation of the question does renal tissue devoid of vasa afferentia and thus devoid of both granular and non granular juxtaglomerular cells contain renin? While *Kaplan & Friedman* studied the renin content of foetal kidneys the present studies were performed on different parts of kidneys of new born pigs In this way a comparison could be made between morphology and renin content of both immature and mature tissue from the same kidney

Supported by grants from *I A Brandt's F n t n* *King Christian X's* Foundation and ...
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MATERIAL AND METHODS

New-born pigs weighing from 700 to 1280 grammes were narcotized with nembutal. The kidneys were removed, weighed and within few minutes after removal either fixed by perfusion with a mixture of saturated ammonium sulphate and light green stain or frozen with carbon dioxide snow, a smaller piece being fixed in Hellys fluid. From the unfixed frozen tissue about 50 µ thick sections were cut on the cryostat. The sections were further microdissected below the stereo microscope with a fragment of a razor blade (Fig. 1). In this way the following preparations were obtained:



Fig. 1
The outer about 70 µ deep subcapsular zone has been isolated by microdissection (400 ×)

A. A 50 to 70 µ broad ribbon of the outermost nephrogenic zone of the kidney, the distance from the surface being determined by a micrometer.
B. A preparation containing the whole cortex.
Other parts of the unfixed fresh frozen tissue was used for histochemical examination, 6 µ thick cryostat sections being stained both for succinate and glucose 6 phosphate dehydrogenase by the cobalt chelating MTT technique (*Pearse* 1960).
The juxtaglomerular granules were stained with Bowie neutral dye obtained from crystal violet and orange G (Gurr).

Renin and Angiotensin Tests

The *renin* assay was performed on extracts performed in glass homogenizers after addition of a small volume of 0.9 per cent sodium chloride. The extracts were injected intravenously to amytal anaesthetized female rats using the method of *Skeggs, Kahn & Marsh* (1953), the pressor response being compared with the response to a standard renin preparation. The result is given in rat units, one rat unit being about 1/40 of a Goldblatt dog unit. In some experiments the extract was incubated with angiotensinase inactivated plasma from nephrectomized rats and the angiotensin formation determined by comparison with synthetic angiotensin.

RESULTS

Morphology

The kidneys of neonate pigs were found to have the same structure as that of several other neonate mammals, having a distinct zoning of maturity of glomeruli, as *Leeson & Baxter* (1957) and *Lewis* (1958) described for the neonate rabbit, *Jokelainen* (1963) for the foetal and

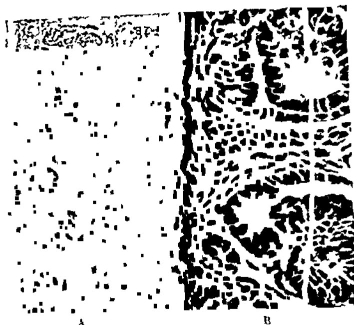


Fig 2

A The outer part of the cortex of a new born pig A line is drawn about 70 μ from the capsule The tissue superficial for this line is very immature containing no arterioles (100 ×)
B Higher magnification (400 ×) of part of the same preparation

neonate rat and *Vernier & Birch Andeersen* (1962) for the human foetal kidney While the juxtamedullary glomeruli were "adult in type, the cortical ones had a thick palisade visceral epithelium and capillaries containing few or no red cells The injected kidneys showed the picture described by *Hartroft* (1941) with adult vascularization of the inner zone of the cortex but only capillaries in the subcapsular tissue, including the 70 μ deep subcapsular zone For the present studies the morphology of this 50 to 70 μ deep subcapsular zone is of special interest as the renin content of isolated ribbons (Fig 1) of this part was compared with the content of the total cortex As seen in Fig 2A and B this subcapsular part contained only very immature tissue containing the blind ends of the collecting ducts covered by caps of metanephrogenic blastemal cells and stromal mesenchymal cells early renal vesicles which in some cases formed S-shaped bodies including preglomerulus (progenitor cells as well of the glomerular epithelium as of the Bowman's capsule) and the pre macula densa containing tubular cells At this early stage of development the tissue lacks both afferent and efferent vessels and is thus absolutely devoid of both granular and non granular epitheloid cells the only vessels in the preglomerulus being fine capillaries formed by the stromal mesenchymal cells which invade the cleft of the S shaped body (Fig 3) The morphology is thus



Figs 5–6

Fig 5 The subcapsular zone is unstained by histochemical reaction for succinate dehydrogenase (100 ×)

Fig 6 The first strong staining reaction for glucose 6 phosphate dehydrogenase is seen about 90 μ from the capsule (400 ×)

the macula densa and that part of it is deposited in the afferent arteriole While most of the granulation is found in epitheloid cells, which are adjacent to the macula densa cells, granulated cells are also found at some distance from the macula The reason for this location which is especially pronounced in hypergranulated kidneys may be, that renin is released into the interstitial fluid and the lymph, as shown by *Lever & Peart* (1962), and thus comes into contact with vessels lying farther from macula

SUMMARY

In studies on kidneys from new-born pigs renin was found in the subcapsular zone which is devoid of afferent and efferent vessels and thus also of both granulated and non granulated epitheloid cells, while it contains tubular tissue including the premacular cells This result confirms previous studies on foetal kidneys by *Kaplan & Friedman* (1942), it further agrees with our previous result of microdissection experiments, in which most of the renin was found in the macula densa containing part of the distal tubule From there renin is believed to get into the vessels either directly or through the interstitial fluid

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Figs 5 6

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FROM THE INSTITUTE FOR GENERELL OG EKSPERIMENTELL PATOLOGI
UNIVERSITETET I OSLO (HEAD LEIV KREYBERG)

THE RESPIRATION OF MOUSE EPIDERMIS AFTER A SINGLE APPLICATION OF 3-METHYLCHOLANTHRENE IN BENZENE

By

OLAV HILMAR IVERSEN and OLE DIDRIK LAERUM

Received 17 vii 63

In a previous paper (*Iversen & Evensen* 1962) the respiration of epidermal slices from hairless mice 24 hours after a single application of methylcholanthrene was reported. A decreased respiration was found, and at the same time we observed an increased amount of formazan deposition as revealed by the the tetrazolium method.

The conclusion was drawn that the carcinogen exerted a destructive effect on the mitochondria of many of the cells.

The intention of the present paper is to extend the study of the oxygen consumption of epidermis after a single application of a carcinogenic compound, *viz* 3 methylcholanthrene.

MATERIALS AND METHODS

Hairless mice of the strain hr/hr were used for the experiments. On the left side of the back skin was dropped 0.005 × 3 ml of a 1 per cent solution of 3 methylcholanthrene (HUkA) on a circumscribed area limited by a special pair of forceps (*Iversen* 1960).

At different time intervals after the painting, the animals were killed by neck fracture, the skins flayed off and a piece of epidermis was removed from the painted area by means of a *Castroviejo Keratotome*. A similar specimen was taken symmetrically from the other side of the back skin on the same mouse serving as control.

The epidermal specimens were floated on a medium consisting of one part of human plasma, one part of Krebs Ringer phosphate, 0.1 part 5 per cent glucose and a little Streptomycin (*Cruickshank* 1954). The O_2 consumption per mg dry epidermis was measured in a Warburg apparatus and the ratio between the values from the painted and from the unpainted areas was calculated for each mouse. The final pH of the medium after one hour of respiration was 7.4.

RESULTS

Table 1 demonstrates the results obtained 2, 4, 8, 16 and 24 hours after the carcinogen application. It is seen that after a short and a slight increase, the O_2-consumption falls down to a lower level, significantly low 24 hours after the carcinogen application.

Fig. 1 shows the results compared to the results reported earlier with the tetrazolium method (*Iversen & Evensen* 1962). It is seen that

where is the formazan deposition in epidermis increases during the first 24 hours after a methylcholanthrene application the O_2-consumption shows a decreasing curve

TABLE 1

The Respiration of Hairless Mouse Epidermis at Different Time Intervals after a single Application of a one per Cent Solution of 3-methylcholanthrene in Benzene The Results Are expressed as the Ratios between the Values Observed from Treated and from Untreated Areas of Epidermis from the same Mouse

Hours after MCA application	No of mice	Ratio treated/untreated	SD of the mean
2	5	1 190	0 22
4	6	1 095	0 03
8	4	1 013	0 10
16	5	0 891	0 32
24	6	0 799	0 07

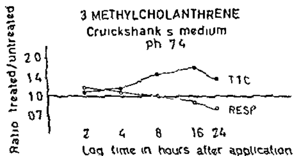


Fig 1

Th
ratios between the values observed from treated and from untreated areas of epidermis from the same mouse

DISCUSSION

It is generally held that a quantitative correlation exists between the the amount of formazan method It seems evident expected when cells are irreversibly damaged Holmberg (1961) has observed that irreversibly damaged cells can give rise to increased deposition of formazan He explains this phenomenon as being the result of an increased permeability of cellular membranes

Our study has confirmed our previous results that after a single application of carcinogens to the epidermis the respiration measured as O_2-consumption per mg dry epidermis decreases during the first 24 hours This may be taken as a further support for our theory that

the carcinogens can destroy the mitochondrial function of epidermal cells

During this work we have, however, observed that the respiration of epidermis is very sensitive to even small alterations of the pH of the medium It seems that not only the respiration of the normal epidermis, but also the changes found after a carcinogen application, can be heavily influenced by variations in the medium-pH The results presented in this paper is, quantitatively, only valid for a medium pH of 7 2 to 7 4

A systematic study of the influence of varying pH on the respiration of the mouse epidermis, combined with a study of the production of lactic acid in the epidermis, after application of carcinogenic and non carcinogenic compounds is in progress at our institute

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FROM THE UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY
(HEAD O TORGERSEN, M D) RIKSHOSPITALET, OSLO, NORWAY

EXCRETION OF PROTEINS FROM THE HUMAN GASTRIC MUCOSA

An Immuno-Histochemical Study

By

BRIGT ØYSTESE and OLAV TORGERSEN

Received 8 vii 63

Paper- and immunelectrophoretical studies have revealed the presence of normal serum proteins in gastric juice, particularly in diseases entailing hypoproteinaemia ("protein-losing gastritis", ulcerative colitis, regional enteritis and gastric cancer) (1–6)

The mechanism whereby the serum proteins escape into the stomach is, however, less well understood The present studies were undertaken in an attempt to trace the pathways of this protein-escape by means of the fluorescent antibody technique

MATERIALS AND METHODS

Preparation of Tissue Sections

The [illegible]
with d [illegible]
pieces [illegible]
tures a [illegible]
infiltr [illegible] vacuum

Fixation

For studies of proteins the sections were fixed for 30 minutes at 37° C in subsequent baths of absolute ethanol and acetone

Antisera

Antihuman albumin was prep[illegible]
by 3 w [illegible]
over a [illegible]
[illegible] The latter was removed by
[illegible] tested
[illegible] intramuscular injections of

The authors are indebted to the Norwegian Cancer Society (Landsforeningen mot Kreft, Oslo) for economical support Appreciation is also expressed to Miss Grethe [illegible] for competent and cheerful technical assistance

Conjugation

The antisera to be conjugated were precipitated at 4° C by 40 per cent ammonium sulphate and washed twice with 50 per cent ammoniumsulphate solution. The precipitate was then suspended in 0.15 M saline and dialysed for 12-24 hours against 0.15 m saline. The final concentration of protein was determined by its absorption at 280 μ m.

To the globulin solution (1 vol) in a concentration of at least 2.5 per cent was added dropwise at 4° C under continuous stirring a solution of 10 mg crystalline FITC (Baltimore Biological Laboratories) per gram protein as determined in 0.2 vol. of cold 0.5 M carbonate buffer (pH 9). The stirring was continued for another 8 hours and the solution was allowed to stand at 4° C overnight. The conjugate was then adjusted to pH 7.5 with 1 N HCl and dialysed for 24 hours against 0.01 M phosphate buffer of pH 7.5 changed at frequent intervals.

2 ml of the conjugate corresponding to about 50 mg of protein was added to a cellulose column which had been equilibrated with 0.01 M phosphate buffer of pH 7.5. This buffer was run through the column until no more fluorescent material emerged. The first fraction thus eluated usually contained low coupled antibodies which gave a specific staining reaction. This reaction, however, was very weak and the fraction therefore discarded. The second and satisfactory fraction was eluated by means of 0.01 M phosphate buffer containing 0.14 M NaCl. It contained labelled proteins which gave a bright fluorescence with negligible non-specific staining. By means of phosphate buffers with higher concentrations of NaCl, high coupled antibodies, which tend to give unspecific staining, were eluated (10, 11).

Staining

Both the direct and the indirect method was used. In the direct method the sections were incubated with conjugated serum for 20 minutes in a moist chamber followed by washing for 10 minutes in three changes of buffered saline of pH 7.5. By the direct method the sections were incubated at 37° C with unconjugated antiserum for 20 minutes, next washed for 10 minutes with buffered saline and then stained with a conjugated sheep antirabbit serum. The conjugates were applied in a concentration of about 1 mg protein per ml buffered saline of pH 7.5. The sections were finally dehydrated, cleared in xylene and mounted in Fluormount (F. Gurr, London).

Controls

The immunological specificity of the staining was tested in a series of inhibition experiments. In blocking tests the sections were incubated with unconjugated samples of homologous antiserum prior to incubation with conjugated antiserum. Additional sections were treated with labelled antiserum that had been adsorbed with homologous antigen in slight excess. Using the indirect method control sections were treated either with unlabelled normal rabbit serum or the primary layer was omitted.

Fluorescence Microscopy

A Zeiss Ultraphot II provided with an Osram HBO 200 light source was used. The sections were examined in dark field and exposures made on a Kodachrome Tri X or a Super Anscochrome daylight film.

RESULTS

When sections were incubated with either antihuman γ-globulin or antihuman albumin an adsorption of conjugate to the mucous substance outlining the superficial zone of the mucosa was observed. Particularly in specimens from gastric cancer strands of brilliant fluorescence outlined the surface epithelium and foveolae and filled the lumina of the glands (Figs. 1 and 2). There was no detectable difference of staining intensity between the neck region and the deeper portions



Fig 1
Albumin in gastric secretion. Section from corpus fixed in subsequent baths of absolute alcohol and acetone and stained with conjugated anti human albumin. Bright fluorescence confined to the lumina of the glands. The patient had gastric cancer × 80

of the glands. Sections from the proximal and distal parts of the resected specimens gave identical results with regard to localization as well as brilliance of staining. A positive reaction was also observed in lymphatics and blood vessels and in some atypical glands in carcinomas. In duodenal specimens a positive reaction was also seen in some of the goblet cells.

Intracellular fluorescence occurred occasionally in the surface and the columnar epithelial cells. It was hard to decide whether or not such findings were due to fixation artefacts only.

Corresponding examinations of material from patients with duodenal ulcer revealed a more variable picture with regard to the amount of secreted proteins. On the average, however, the staining intensity was



Fig 2
A similar section from the surface of the gastric mucosa × 160

distinctly lower, while localization of fluorescence was similar to the findings in gastric cancer

As previously described, the specificity of staining was ascertained by a number of control tests Negative results were obtained in all of these, except for the blocking test, where there was a distinctly reduced intensity of staining, instead of complete inhibition This was probably due to excess of antigens in the tissue section

DISCUSSION

For a demonstration of excreted proteins the tissue fixation constitutes a problem Since incubation is carried out with an aqueous antibody-solution, insufficiently fixed proteins may be dissolved into the medium before the actual immunological reaction has taken place By the subsequent antigen antibody reaction these proteins may be precipitated diffusely on the tissue section and may lead to errors of localization Thus, after treatment with either absolute ethanol methanol, acetone or formalin vapor, a halo of fluorescent material could be seen partly surrounding and partly covering the sections Metallic fixatives (5 per cent $HgCl_2$ or 1 per cent osmic acid, both in ethanol) gave a better localization, but at the same time tended to quench the fluorescence Subsequent baths of absolute ethanol and acetone for 30 minutes each, at 37° C, gave the best fixation, and was therefore adapted to routine usage

With regard to the histological demonstration of serum proteins in the excreted gastric mucus, the findings are in agreement with results of paper and immunelectrophoretical studies of gastric juice (1 6) Since the proteins were demonstrated to fill the entire lumens of the foveolae as well as those of some glands, it was unlikely that they derived from either contamination of saliva or minute haemorrhages These observations are considered to support the idea of an actual excretion of proteins from gastric mucosa

Concerning the topographic distribution of the demonstrated proteins no difference could be observed between sections taken from the proximal and the distal parts of the resected specimens The findings, however, indicate a higher content of excreted proteins in cases of gastric cancer, as compared with patients with gastric or duodenal ulcer

In contrast to the findings with regard to bloodgroup substance A, we have so far been unable to trace the conditions preceding the excretion of albumin and γ globulin This lack of a convincing demonstration of the proteins in question within the cytoplasm of the gastric epithelial cells may be due to several factors

a) the proteins may be transported intercellularly,
b) the proteins may be transported through the *epithelial cells, but* in such low concentrations that they *are not traceable by the* fluorescenceimmunological method,
c) *the proteins* present within the cells may be bound to cytoplasmic components (muco proteins) so that they are immunochemically masked

SUMMARY

In resected and biopsy material from human stomachs large amounts of excreted serum proteins (*albumin* and γ-globulin) were demonstrated in the mucous substance outlining the epithelial surface of human gastric mucosa These proteins were also located in the entire lumina of the crypts as well as in some atypical glands in cancer tissue By the method used the proteins could not be demonstrated with certainity in the cytoplasm of the epithelial cells

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FROM THE DEPARTMENT OF SURGERY AND DEPARTMENT OF CLINICAL BIOCHEMISTRY, ROYAL VETERINARY COLLEGE, STOCKHOLM, SWEDEN

RENAL FUNCTION IN DOGS WITH PYOMETRA

8. *Uterine Infection and the Pathogenesis of the Renal Dysfunction*

By

ÅKE ÅSHEIM

Received 14.x.63

Polydipsia and polyuria commonly accompany pyometra in bitches (*Canine Medicine* 1959, *Åsheim* 1963). The renal dysfunction represents a reduction in the concentrating ability of the kidneys, usually together with a reduction in glomerulus filtration (*Åsheim* 1963, 1964 a, b). The severity of these two components in the renal dysfunction seems to vary independently (*Åsheim* 1964 a, d). The reduction in the glomerular filtration rate seems to be caused by morphological changes in the glomerulus (*Obel et al.* 1964). A morphological background to the reduction in concentrating ability which accompanies pyometra could not be demonstrated with certainty.

The initial phase in the pathogenesis of pyometra is apparently hormonal dysfunction resulting in endometrial hyperplasia (*Teunissen* 1952, *Dow* 1959). Endometritis can subsequently develop and bacteria can then generally be demonstrated in the uterine contents (*Dow* 1958). Bacterial invasion probably occurs at oestrus.

The possibility of an association between uterine infection and renal dysfunction prompts the questions:

1. Are the bacteria in the uterine contents to be considered pathogenic or simply as saprophytes?
2. Is the presence of bacteria in the uterus a prerequisite for the renal dysfunction?
3. If this is the case, how do the bacteria in the uterus affect renal function?

These three questions will be dealt with in turn.

1. ARE THE BACTERIA IN THE UTERINE CONTENTS TO BE CONSIDERED PATHOGENIC OR SIMPLY AS SAPROPHYTES?

One sign that microorganisms have actually infected the host, *i.e.* elicited a host response, is the induction of specific antibodies.

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Staphylococci + streptococci	3
No growth	6

Three of the six bitches from which no organisms could be isolated had previously been treated with antibiotics or sulphonamides As far as could be ascertained, none of the bitches from which bacteria were isolated had received such treatment

Discussion

Bacteria are practically invariably present in the uterus of pyometra bitches with reduced concentrating ability The negative results for six of the bitches do not exclude the possibility that bacteria had previously been present, three of these bitches, at least had been treated with bacteriostatics Furthermore, there was histological evidence of neutrophil infiltration of the endometrium, another sign that bacterial infection had probably occurred, in *all* the bitches

B *Renal Dysfunction and the Uterine Bacterial Flora in Experimentally Induced Pyometra*

To follow renal function and changes in the uterus, pyometra was induced in bitches by *Dow's* (1959) method—injection of ovarioectomized animals with stilboesterol and progesterone

Material and methods

The ovaries were removed from five normal bitches 1 to 3 years old, and the [illegible] days and 25 mg [illegible] was commenced [illegible] intake and urine [illegible] followed during experiment

Blood studies included white and red cells counts the erythrocyte sedimentation rate (FSR) haemoglobin content haematocrit and non protein nitrogen (NPN) Sodium and potassium [illegible]
osmolarity by [illegible]
The protein co[illegible]
The protein co[illegible]
pH 8 9 in a LK[illegible]
(cam B 5) [illegible]

Urine studies included examination of the sediment, determination of urine osmolarity (Uosm) by freezing point depression, and examination for the presence of glucose and protein
[illegible]

[illegible] microscopically

Results

One (bitch P2) of the five bitches developed a brownish discharge from the vulva in the third cycle during progesterone treatment, 81

Material and methods

These studies were carried out on sixteen bitches with clinical signs of pyometra and in which E coli was isolated from the uterus contents The diagnosis of pyometra was confirmed by examination of the uterus after ovariohysterectomy Samples of the uterus contents were obtained for bacteriological examination at the time of ovariohysterectomy and blood serum samples were taken immediately before operation Serum from normal bitches was taken for comparison

Precipitation tests were run on the serum samples A five day old broth culture of the specific E coli strain was used as antigen and tested against the serum from the bitch from which the particular strain had been isolated For titration the serum was diluted with serum from normal bitches

Results

Positive precipitation reactions in dilutions up to 1 8 were obtained for all sixteen pyometra bitches The same E coli antigens gave no reaction with serum from dogs infected with streptococci Nor did they react with serum from normal bitches

Conclusion

That the E coli strains isolated from the uterus had incited the formation of antibodies in their host is a sign that the bacteria are pathogenic

2 IS THE PRESENCE OF BACTERIA IN THE UTERUS A PREREQUISITE FOR THE RENAL DYSFUNCTION?

A *Bacteriological Examination of the Uterus of Pyometra Bitches with Reduced Concentrating Ability*

Material and methods

Seventy nine bitches with increased thirst accompanying pyometra were available for examination The polydipsia had been observed up to 15 days (mean 3 days) before the animals were submitted to the clinic A few of these bitches had had increased thirst for several months but a further increase occurred concomitantly with the other manifestations of pyometra The renal function tests carried out on these animals have been dealt with elsewhere (*Asheim* 1963 1964 a b c d) All bitches had a reduction in the concentrating ability The mean max osmotic *U/P* ratio was 1 9 (normal 5 4) with a range of 1 0 to 3 9 Samples of the uterus contents for bacteriological examination were taken at the time of ovariohysterectomy The clinical diagnosis of pyometra was confirmed by examination of the excised uterus

Results

The following organisms were isolated from the uterus

E coli	64
Staphylococci	2
Streptococci	4

The serological examinations were carried out by laborator *P Virden* State Veterinary Medical Institute Stockholm

Text) of Experimentally Induced Pyometra in Bitch No P2

	Serum protein											
	Albumin		Globulin									A/G ratio
			Alpha 1		Alpha 2		Beta		Gamma			
	rel %	g 100 ml	rel %	g 100 ml	rel %	g 100 ml	rel %	g 100 ml	rel %	g 100 ml		
8	49 5	2 9	4 9	0 3	11 4	0 7	18 0	1 1	16.3	0 9		0 98
6 8	47 4	3 2	4 0	0 3	14 6	1 0	18 6	1.3	15 4	1 1		0 86
8 1	25 9	2 1	5 3	0 4	17 3	1 4	14 4	1 2	37 0	3 0		0.35

Discussion

The onset of naturally occurring pyometra and experimentally-induced pyometra is probably associated with an unusually high progesterone level in the blood Theoretically, the renal concentrating ability can be influenced by progesterone since this hormone has aldosterone-inhibiting properties with a natriuretic effect as a result (*Landau & Lugibihl* 1958) Increased sodium excretion can be an explanation for the reduction in the sodium content in the renal medulla which can be demonstrated in pyometra (*Asheim* 1964 c)

The results presented here, however, suggest that it is infection and not hormone unbalance which is basically responsible for the reduction in concentrating ability Only the bitch with uterine infection acquired polydipsia and polyuria There is, of course, the reservation that only a few animals were covered by this study

3 HOW DO BACTERIA IN THE UTERUS AFFECT RENAL FUNCTION?

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A Bacteriological Examination of Kidney Tissue

Bacterial infection of the kidneys, as is the case with pyelonephritis in human beings can lead to reduction in maximum urine osmolarity (*Jackson et al* 1957, *Bricker et al* 1959, *Kleeman et al* 1960) *E coli* is most commonly isolated from human beings (*Kleeman et al* 1960, *Brod* 1962) Since this organism accounts for most of the isolates from pyometra bitches, spread of the infection from the uterus to the kidneys

TABLE

Blood and Serum Protein Values before and at Various Stages

Treatment days	Blood							Plasma		
	White 10^3	Red 10^6	Hb g 100 ml	Hcrit vol %	ESR mm hour	NPN mg 100 ml	CO_2 vol %	Osm mOsm l	Na mEq l	K mEq l
0	9 3	5 6	15 0	44	3	24	54	293	148	41
82	8 4	5 1	11 8	48	9	27	54	290	150	43
95	25 6	2 9	8 2	25	134	24	44	288	138	39

days after hormone treatment was begun The amount of discharge decreased only to increase again during the fourth cycle The bitch died in the middle of this cycle, after 107 days of hormone treatment

Max osmotic U/P ratio was 6 5 before hormone treatment was begun The ratio dropped when the discharge appeared and ultimately reached a low of 1 5 At the same time, urine volume and thirst increased The normal water intake of about 400 ml per day gradually rose to 2,000–3,000 ml per day

The uterus was normal in size on radiograms taken 57 and 69 days after the beginning of the experiment By 83 days, the uterus was about 1 cm in diameter and by 95 days had a diameter of about 3 cm At autopsy the uterus had a diameter of about 3 cm The autopsy diagnosis was pyometra Abundant growth of streptococci and staphylococci was obtained from the uterine exsudate

The clinical appearance of the bitch was compatible with pyometra When the discharge commenced the animal was depressed and had a poor appetite She vomited repeatedly the days preceding death The laboratory results are listed in Table 1 The changes in the blood and serum proteins were similar to those reported by *Freudiger* (1955) and *Eikmeier & Moegle* (1959) for pyometra and those observed in my own investigations on clinical cases

On day 95 the urine contained bacteria and a few white and red cells No protein could be detected

The other four bitches remained healthy during the 5 to 7 months of hormone treatment after ovarioectomy Concentrating ability and water intake varied within normal limits There were no pathological changes in the blood or urine The uterus in all four bitches was somewhat enlarged at the end of the experiment Histologically the endometrium was slightly to moderately hyperplastic and infiltrated by plasma cells and lymphocytes

No bacteria could be isolated from the uterine contents

Results

The bitches reacted to the first injection with uneasiness, tremor, ataxia, emesis and high fever. They soon developed a tolerance, however, and generally regained their appetite and appeared unaffected by subsequent injections. If the dose was increased greatly, the clinical signs reappeared temporarily only to subside within a few days as tolerance was again established. There were also local reactions at the site of injection. These regressed fairly rapidly in all but one bitch (F7) in which large patches of the skin were involved.

The effect of the toxin injections on concentrating ability is given in Table 2. All bitches experienced a reduction; the largest doses of toxin resulted in the greatest reductions. Bitch F8 received subcutaneous injections of toxin for 64 days and for the last eight days the daily dose was 20 ml. The lowest osmotic U/P ratio attained was 3.9. From days 65 to 69, 20 ml were given intravenously and 20 ml subcutaneously. The U/P ratio declined to 2.9.

All the bitches except F5 and F12 increased their water intake and urine volume. During the later part of the experimental period bitch F7 drank between 1500 and 1900 ml per day (initially about 400 ml). During the first 64 days bitch F8 increased her daily water intake from some 400 ml to about 700 ml. When the toxin dose was doubled, water intake increased to about 2000 ml.

Three to four weeks after the toxin injections ceased, the concentrating ability for three of the bitches was tested again. All three had regained normal values.

Discussion

Injection of E. coli toxin induced a reduction in concentrating ability comparable with that which is commonly associated with pyometra. The concentrating ability remains reduced even after the animal has acquired tolerance to the systemic effects of the toxin. Increasing the dose of toxin can break through the tolerance; systemic effects are again manifest and the concentrating ability is reduced still further. This observation tallies with the results of the experiment described under 2 B and clinical observations (unpublished) that rapid enlargement of the uterus—and presumably rapidly increased release of toxin—usually leads to an immediate deterioration of the general condition and further reduction in concentrating ability.

Reduction in the concentrating ability in pyometra bitches also accompanies infection with streptococci and staphylococci. Release of toxin is probably responsible in such instances as well since toxin activity seems to be independent of bacterial species (*Bennet & Cl*...

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—if, in fact, this occurs—could conceivably result in a reduction in concentrating ability

Material and methods

Kidney tissue obtained in conjunction with ovariohysterectomy by the biopsy technique described previously (*Obel et al* 1964) from fourteen pyometra bitches with E coli infection in the uterus was examined bacteriologically

Results

In no instance could infection be demonstrated in the kidney tissue

B *Renal Function and E coli Toxins*

The injection of dogs with a bacterial pyrogen in sublethal doses results in diuresis because of reduction in the concentrating ability of the kidneys This phenomenon can be elicited even in the presence of antidiuretic hormone (*Brandt et al* 1955) and, according to *Hinshaw et al* (1959, 1961), results from increased blood flow through the kidney tissue and not from a toxic effect on the tubules The increased blood flow probably "washes out" sodium ions to reduce the hypertonicity of the renal medulla and the ability to resorb water (*Elpers & Selkurt* 1963)

Materials and methods

E coli toxin was prepared (by laborator *P Virden* State Veterinary Medical Institute) from a strain isolated from pyometra bitch 95/62 After three days in Roux flasks containing beef broth agar the cultures were washed three times in physiological saline solution The bacteria in each flask were suspended in 80 ml saline solution the suspension heated at 80° C for 30 minutes centrifuged and passed through a Zeiss filter The filtrates were used for injection

TABLE 2

Dosage of E coli Toxin and Max Osmotic U/P Ratio before and during Exposure to Toxin (Lowest Value Obtained)

Bitches no	Toxin ml day	Day no	Administration	Max osmotic U P ratio	
				Before administration	During administration
F5	Gradually increased to 5 ml	49	s c	6 4	5 0
F6	Gradually increased to 5 ml	43	i v	5 8	3 2
F7	20 ml	48	s c	7 0	3 8
F8	Gradually increased to 20 ml	64	s c	6 2	3 9
	2 × 20 ml	65 69	s c + i v		2 9
F12	1 ml	93	s c	5 2	4 0
F13	10 ml	93	s c	5 8	4 5

s c = subcutaneously i v = intravenously

The filtrates were injected daily into six normal bitches 1 to 4 years old The amounts injected are listed in Table 2 During the experiment daily water intake and urine volume were followed and maximum concentrating ability was checked after dehydration for 24 hours and the administration of antidiuretic hormone

Results

The bitches reacted to the first injection with uneasiness, tremor, ataxia, emesis, and high fever They soon developed a tolerance, however, and generally regained their appetite and appeared unaffected by subsequent injections If the dose was increased greatly, the clinical signs re-appeared temporarily only to subside within a few days as tolerance was again established There were also local reactions at the site of injection These regressed fairly rapidly in all but one bitch (F7) in which large patches of the skin were involved

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Discussion

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[illegible] how the toxin exerts its effect upon the concentrating ability remains unsolved It is conceivable that the reduction in the sodium gradient in the renal medulla which occurs in pyometra (*As-*

heim 1964 c) results from a vascular effect on the kidney induced by the toxin It is also possible that the toxin renders the epithelium of the distal tubules unresponsive to ADH, a phenomenon known to occur in pyometra (*Asheim* 1964 b)

In summing up we must take into account that bacterial infection can be demonstrated in the uterus of the great majority of pyometra bitches with reduction in the renal concentrating ability The bacteria incite the formation of specific antibodies, a sign that they are pathogenic Injection of toxin prepared from an E coli strain isolated from a pyometra bitch resulted in reduction of the concentrating ability This observation offers an explanation for the polyuria and polydipsia commonly associated with pyometra

SUMMARY

Bitches with pyometra have circulating antibodies specific for the bacteria in the uterus

Bacteria were isolated from the uterus of 73 of 79 pyometra bitches examined The histopathological appearance of the endometrium indicates that infection had occurred even in the bitches from which no organisms could be recovered

Attempts to induce pyometra experimentally by hormone injections resulted in reduction of the renal concentrating ability in the one of five bitches in which the uterus became infected No signs of renal dysfunction were noted in the other four bitches exposed to hormonal effects only

Injection of toxin prepared from an E coli strain isolated from a pyometra bitch induced a reversible reduction in renal concentrating ability

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FROM THE DEPARTMENTS OF BACTERIOLOGY AND PHARMACOLOGY
ROYAL VETERINARY COLLEGE, STOCKHOLM 50, SWEDEN

FURTHER PATHOPHYSIOLOGICAL INVESTIGATIONS INTO THE TERMINAL COURSE OF EXPERIMENTAL ANTHRAX IN THE RABBIT

By

B. K. Nordberg, C. G. Schmiterlow, B. Bergrahm and H. Lundström

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In a previous investigation (*Nordberg, Schmiterlow & Hansen* 1961) we showed that the terminal course of experimental anthrax in the rabbit can be characterized as an asphyxia associated with an extremely advanced haemolysis. The haemolysis, although not the only cause, is probably one of the factors responsible for this asphyxia.

The present paper deals with some other aspects in connection with the terminal phase of experimentally induced anthrax infection in rabbits.

METHODS

Throughout this investigation rabbits weighing between 2.4–2.6 kg have been used. The animals were infected by intravenous injection of a sporulating culture of *B. anthracis* totaling 10–13 × 10^7 spores. The infective agent was a mixed culture from 5 highly virulent strains of *B. anthracis* that have been isolated from Swedish cases of anthrax.

The concentration of blood gases was determined according to the *van Slyke* method using blood samples drawn from the carotid artery by means of a permanent cannula inserted under local anaesthesia. Samples were taken both before the infection to check the normal values determined in our previous paper and at various stages of infection. A special interest was devoted to the O_2 and CO_2 contents in the arterial blood of animals showing clinical signs of having reached the preagonal stage (i.e. 2–5 minutes before death). For the sake of comparison the blood gases were also determined in venous post mortem blood.

Such blood gas analyses were carried out in two groups of infected animals.

One group consisted of rabbits which were given benzyl penicillin (100 000 I.U. per kg body weight intramuscularly) when capsulated bacilli occurred in their blood.

Another group consisted of rabbits which had been splenectomized 8 weeks before the actual anthrax experiment.

Blood sugar analyses were carried out in infected rabbits using the Somogyi-Nelson method.

The pH of the blood from infected animals was determined with a Radiometer pH meter, type TTT 1 A.

Plasma Na^+ and K^+ were determined using a flame photometer (Eel).

This investigation was financially supported by *Medicinska Forskningsrådet* and *Jordbrukets Forskningsråd*.

In a series of experiments the blood pressure was recorded from the carotid artery. The cannula was inserted under local anesthesia 2-6 hours before the expected death of the anthrax infected rabbits.

RESULTS

Content of Blood Gases in Anthrax Infected Rabbits Treated with Penicillin

As we have pointed out earlier (*Nordberg, Schmiterlow & Hansen* 1961, *Schmiterlow & Nordberg* 1962) such anthrax infected rabbits that were given penicillin before, or soon after, the culmination of the rise in temperature recovered, whereas rabbits treated at a later stage when the microbes in their blood had assumed the capsulated form did not survive. In the present series of experiments we have determined the content of blood gases in rabbits given penicillin at such a time during the course of the infection that they did not survive. From Table 1 it can be seen that both the O_2- and CO_2-content in the blood assumed approximately the same values as those described earlier by *Nordberg, Schmiterlow & Hansen* (1961) in rabbits succumbing from anthrax without treatment with penicillin. When the blood from these penicillin-treated rabbits was examined microscopically no microbes in the vegetative state could be found. An abundance of capsular substance could, on the other hand, be observed in the blood smears.

TABLE 1

Content of Blood Gases in Anthrax Infected Rabbits Treated with Penicillin

Rabbit no.	Arterial blood				Venous blood	
	O_2 (Vol %)		CO_2 (Vol %)		O_2 (Vol %)	CO_2 (Vol %)
	p. i.	p. a. s.	p. i.	p. a. s.	p. m.	p. m.
11/(2	13.6	0.6	46.3	18.0	0.3	27.6
12/62	15.3	0.4	39.8	19.9	0.3	22.3
15/62	11.6	0.5	41.7	29.8	0.4	29.0
17/62	14.4	0.7	54.8	12.3	0.5	26.6

Abbreviations: p. i. = pre infectionem
p. a. s. = pre agonal stage
p. m. = post mortem

Content of Blood Gases in Splenectomized Anthrax-Infected Rabbits

In order to elucidate whether the classical anthrax sign, splenomegaly, had any influence on the course of the infection rabbits were splenectomized 8 weeks before the actual anthrax experiment. From Table 2 it is obvious that the splenectomized rabbits behaved exactly like the normal anthrax infected rabbits, *i.e.* they showed an extremely low O_2-content both in arterial blood in the pre-agonal stage and in the venous blood taken after death. Obviously the absence of the spleen did not interfere with the course of the infection.

TABLE 2

Content of Blood Gases in Anthrax Infected, Splenectomized Rabbits

Rabbit no	Arterial blood				Venous blood	
	O (Vol %)		CO (Vol %)		O (Vol %)	CO (Vol %)
	p i	p a s	p i	p a s	p m	p m
16	11 3	0 1	44 2	20 8	0 1	16 2
17	13 6	0 2	38 1	20 2	0 1	26 8
18	10 7	0 1	37 1	27 6	0 1	20 7
19	13 9	0 3	36 0	26 0	0 2	30 0

Abbreviations Same as in Table 1

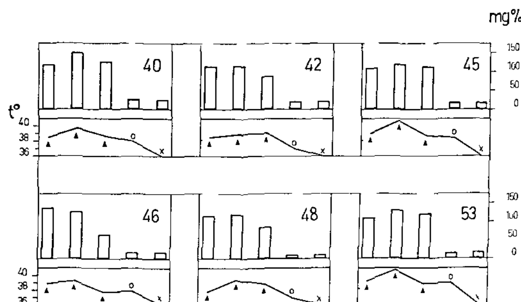


Fig 1

Blood sugar in rabbits at various stages of anthrax infection

Symbols ▲ = Samples taken during the infection
○ = Samples taken in preagonal stage
× = Samples taken post mortem
t° = Body temperature

Blood Sugar Content

Bloom et al (1947) described disturbances in carbohydrate metabolism during the course of experimentally-induced anthrax in rabbits In 14 out of 21 rabbits there was a marked hyperglycaemia, the peak occurring usually 8 to 12 hours before death Only one animal showed hypoglycaemia whereas the other 6 showed no significant variation from the normal

In our experiments we have found that the blood sugar level shows a slight elevation in the earlier stage of anthrax This elevation seems to parallel the rise in body temperature Simultaneously noncapsulated

anthrax bacilli begin to occur in the blood When the body temperature starts to sink the blood sugar also begins to fall At this stage capsulated bacilli begin to occur in increased number in the blood In the pre-agonal stage a marked hypoglycaemia was observed The results are summarized in Fig 1

The blood sugar content was also determined in rabbits which were mechanically strangulated under general anaesthesia In these cases a marked hyperglycaemia was observed

Determinations of Blood pH

The pH of whole blood samples was determined throughout the course of the anthrax infection The results are shown in Table 3 in which the pH values are correlated with the body temperature at the time when the samples were taken

TABLE 3

Blood pH Values in Anthrax Infected Rabbits

Rabbit no		Temperature C	pH values	Rabbit no		Temperature C	pH values
13	p i	38.9	7.70	16	p i	38.9	7.75
		40.8	7.75			40.5	7.85
		39.3	7.80			40.2	7.75
	p a s	38.0	7.10		p a s	37.0	7.40
	p m		6.85		p m		6.80
14	p	38.6	7.70	17	p i	38.7	7.70
		40.5	7.90			41.0	7.90
		38.9	7.85			39.9	7.70
	p a s	38.8	7.50		p a s	37.8	7.20
	p m		7.00		p m		6.90
15	p	38.6	7.70				
		41.0	7.80				
		39.1	7.70				
	p a s	37.8	7.55				
	p m		7.00				

Abbreviations Same as in Table 1

From Table 3 it can be seen that the pH values initially show a tendency to rise but that they later on tend to fall Especially in the preagonal stage low values are encountered indicating an acidosis

Na and K Content of the Blood

The results from these determinations are shown in Fig 2 As can be seen the variations in Na content are within physiological limits during the entire course of anthrax infection The K values on the other hand show a marked increase in the pre agonal stage and continue to rise postmortally

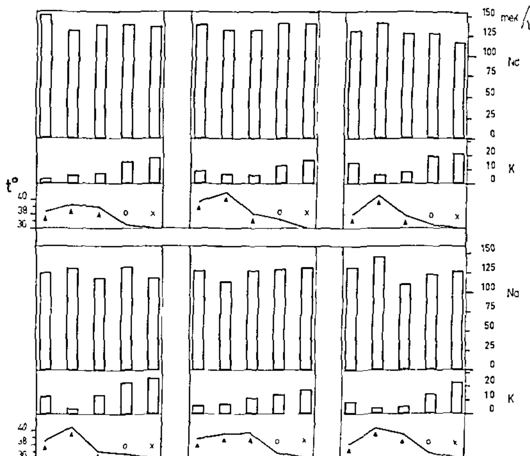


Fig 2
Na^+ and K^+ content in rabbit blood at various stages of anthrax infection
Symbols Same as in Fig 1

Blood Pressure

The blood pressure, recorded from the carotid artery in anthrax infected rabbits, usually showed a fairly constant level during the last 2–6 hours before death Sometimes there was a continuous but slight fall in blood pressure, sometimes there was a slight increase in blood pressure during the last 10 minutes before death Finally the blood pressure picture always presented an abrupt fall when death occurred Figs 3 and 4 demonstrate two typical blood pressure recordings

DISCUSSION

According to *Smith & Keppie* (1955) the removal of the anthrax infection in guinea-pigs by streptomycin saves the animals provided the bacteraemia has *not increased* beyond a certain value All guinea-pigs having a bacteraemia greater than this critical value died 1 3 days later although free from infection *Nordberg, Schmiterlow & Hansen* (1961) and *Schmiterlow & Nordberg* (1962) showed that penicillin treatment

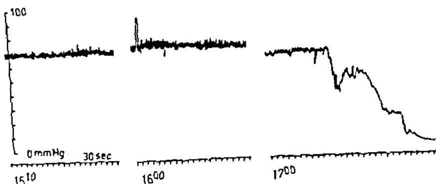


Fig 3

Blood pressure recording from an anthrax infected rabbit showing the maintenance of the blood pressure until the time of death

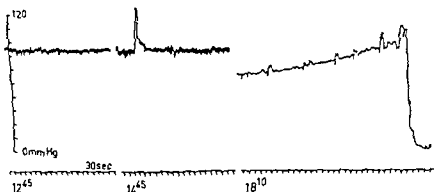


Fig 4

Blood pressure recording from an anthrax infected rabbit Note the gradual elevation in pressure before the abrupt fall at the time of death

of anthrax infection in rabbits was effective as long as the anthrax bacilli occurred in the noncapsulated form in the blood When penicillin was given at a later stage, *i e* when the bacilli had started *to occur in* the capsulated form, the animals died In experiments with S^{35} labelled *anthrax* bacilli (*Bonventre, Nordberg & Schmiterlow* 1961) it was shown that penicillin treatment in mice at an early *stage of anthrax* infection caused a rapid destruction of the microorganisms In the

... anthrax infection Furthermore, the unusually low O_2-content in the blood observed in the pre agonal stage of untreated anthrax rabbits was found to occur even in these penicillin treated cases *It* should be pointed out that blood samples from these penicillin treated rabbits contained no bacilli in the vegeta

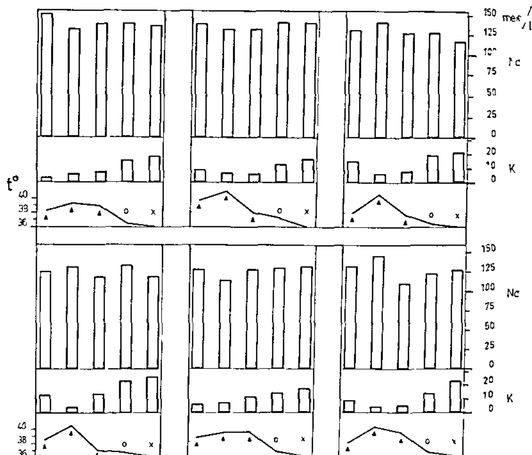


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Na^{+} and K^{+} content in rabbit blood at various stages of anthrax infection
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DISCUSSION

According to *Smith & Keppie* (1955) the removal of the anthrax infection in guinea pigs by streptomycin saves the animals provided the bacteraemia has not increased beyond a certain value All guinea pigs having a bacteraemia greater than this critical value died 1 3 days later although free from infection *Nordberg, Schmiterlow & Hansen* (1961) and *Schmiterlow & Nordberg* (1962) showed that penicillin treatment

animal dies These results are in contrast to the results described by *Smith & Keppie* (1955) who state that: "Direct cannulation of the carotid artery under local anaesthesia showed the blood pressure to be 85, 56, 39 and 18 per cent of the normal value at approximately 9, 6, 1½ and ½ hr, respectively, before death from untreated anthrax". As in our experiments, using rabbits instead of guinea-pigs, we have not found evidence of a gradual fall in blood pressure we find it difficult to believe that rabbits infected with anthrax die from "secondary shock", one of the characteristics of this shock being a drop in blood pressure

As to the real mechanism involved in the terminal stage of anthrax and the direct cause of death there are still many unsolved questions *Smith & Keppie* (1955) hold the view that death from anthrax is a death from secondary shock We agree that some of our findings, such as the pronounced hypoglycaemia, acidosis, and elevation of plasma K^+, are consistent with this view, whereas *e g* the maintenance of the blood pressure until the very moment of death is less compatible with the concept of shock We think that the elucidation of the real mechanism behind anthrax death is more important than the choice of terminology

The extremely low O_2 content of the blood from rabbits dying from anthrax is still impossible to explain An O_2 content of less than 1 per cent is certainly incompatible with life and still the rabbits continue to live for 5 10 minutes It could be argued that our *van Slyke* analyses are in error because the anthrax bacilli present in the blood samples would consume the oxygen during the time that necessarily elapses between the taking of the blood sample and the actual determination of blood oxygen in the *van Slyke* apparatus Against this argument we can only call attention to the fact, described in this paper, that the same low oxygen values were found in blood from penicillin-treated rabbits where no bacilli were present

In a recent paper *Smith & Keppie* (1962) have pointed out the fact that we did not determine the blood oxygen content in anthrax-infected rabbits treated with penicillin In the present paper we have now shown that even in such rabbits the oxygen content is extremely low in the terminal phase of anthrax infection In these rabbits no microbes in the vegetative state could be found in the blood It seems evident that the reason for the low oxygen values which we have described in our earlier publications could thus not be the presence of bacilli in the blood as suggested by *Smith & Keppie* Recently *Eckert & Bonventre* (1963) have studied the effects of B antracis filtrates obtained from *in vitro* grown cultures When such filtrates were injected into albino rats a marked haemoconcentration and an increased erythrocyte fragility, an extremely low oxygen concentration in the blood, and a terminal hypoglycaemia were observed These findings, relating to an anthrax "lethal factor", are in accordance with our findings

tive form This fact strongly indicates that the presence of bacilli in the blood samples taken for *van Slyke* analysis is not responsible for the disappearance of O_2 from the blood samples outside the body (rapid consumption of oxygen by the bacilli) The same abnormally low O_2 content was found also in the blood samples from the penicillin treated rabbits where no bacilli were present

Our findings thus indicate that the low O_2-values are due not to the presence of the anthrax bacilli but rather to some other factor, perhaps the occurrence of capsular substance in the blood This view is principally in accordance with the statement made by *Smith & Keppie* (1955), discussing the effect of streptomycin treatment "This finding seems to render untenable the hypothesis that death from anthrax is due to the bacteria blocking the capillaries or producing a deficiency in the host of O_2 or essential nutrients (*e g* glucose)"

The last statement in this quotation brings us to discuss our finding that in the terminal phase of anthrax there is a marked hypoglycaemia This is in contrast to the observations made by *Bloom et al* (1947) From the description in the paper by *Bloom et al* it is, however, not clear whether the blood sugar level was determined in the pre-agonal stage of anthrax, the marked hypoglycaemia not occurring until this late stage *in our experiments* The statement by *Smith & Keppie* quoted above is not necessarily in conflict with our conception of the pathophysiological events occurring in the terminal course of anthrax Even in our experiments there is no real fall in blood sugar level during the period of infection when the anthrax bacilli could be supposed to use glucose for their metabolism or production of capsular substance The hypoglycaemia does not become marked until the pre-agonal stage where other mechanisms may be involved The findings of *Green & Stoner* (1954) that in shock, by which word they mean "the general reaction of the body to injury", there is an excessive destruction of carbohydrate seem to be consistent with our results Our figures correspond to those given by *Stoner et al* (1952) who found a rise of 30 per cent in the plasma glucose in the early part of the bacteraemia and a reduction of 65 per cent in animals at death It is not unlikely that as a result of the terminal anoxia the blood glucose level falls

As regards the pH of the blood and the plasma Na and K^+ values, our findings are in accordance with those of *Smith & Keppie* (1955) The marked rise in K^+ is most probably due to the excessive haemolysis which we have described earlier (*Nordberg, Schmiterlow & Hansen* 1961) Our figure for haemolysis was definitely much higher than that given by *Smith & Keppie* but it should be noticed that their figure refers to anthrax-infected guinea-pigs, ours to rabbits

Our blood pressure experiments show that even shortly before death the anthrax-infected rabbits are capable of maintaining an almost normal blood pressure and sometimes even show a slight elevation The profound and drastic fall in blood pressure does not occur until the

FROM THE BACTERIOLOGICAL DEPARTMENT OF SERAFIMERLASARETTET
STOCKHOLM SWEDEN (HEAD ARNE LITHANDER)

THE PASSAGE OF PARENTERAL PENICILLIN INTO THE CEREBROSPINAL FLUID IN STAPHYLOCOCCIC MENINGITIS

II An Experimental Investigation on Rabbits

By

ARNE LITHANDER and BRITA LITHANDER

Received 4 xi 63

In a previous report (1), *Lithander & Lithander* studied the passage of penicillin into cerebrospinal fluid and brain in experimental staphylococcic meningitis in rabbits This investigation revealed that the penicillin concentration in both cerebrospinal fluid and brain substance varied with the penicillin concentration in plasma The concentration in cerebrospinal fluid varied also with the intensity of the clinical sings of meningitis

The penetration of the blood cerebrospinal fluid barrier in man by penicillin is said to be relatively slow *Boger & Wilson* (2) reported that approximately two hours are required for penicillin to pass into cerebrospinal fluid following intravenous administration of 500 000 *units in* patients with cerebral syphilis They also stated that penicillin rapidly disappears from cerebrospinal fluid in meningeal infection

Nakamura et al (3) found a very high penicillin concentration in cerebrospinal fluid from the cisterna magna following intracarotid arterial injections of penicillin in patients with otogenous meningitis The maximum was reached 30 minutes after the injection The concurrent concentration in the blood was lower Penicillin could still be recovered in the cerebrospinal fluid two and a half hours after the injection The concentration in cerebrospinal fluid was much lower following [illegible]

and [illegible]

rabbi [illegible]

strep [illegible] when the injection of [illegible]

made in the [illegible]

[illegible] found in the cerebrospinal fluid

Following intravenous injection the concentration of penicillin in the

Further experiments aiming at explaining the cause of death from anthrax in rabbits are in progress in our laboratories

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TABLE 1

The Level of Penicillin in Plasma, Cerebrospinal Fluid and Brain Substance and the Concentration of Leucocytes in Cerebrospinal Fluid in Rabbits with Staphylococcic Meningitis at Different Intervals after Intravenous Administration of Penicillin

		15 minutes		30 minutes		60 minutes		90 minutes		120 minutes		150 minutes		σ	F[1]
		N	Log	N	Log	N	Log	N	Log	N	Log	N	Log		
Plasma	Experiment	6	3.075	30	2.297	6	1.636	7	1.292	7	0.869	5	0.668	0.417	35.75*
	Control	5	3.101	32	2.333	5	1.728	5	1.006	5	0.492	–	–	0.259	97.87*
	F		0.02		0.16		0.20	–	2.14	–	0.81	–	–	–	–
Cerebrospinal fluid	Experiment	6	2.010	30	1.415	6	1.167	7	0.891	7	0.864	5	0.872	0.349	11.36*
	Control	5	0.984	32	0.632	5	0.468	5	0.300	5	0.372		–	0.173	14.36*
	F		40.02*	–	107.46*	–	18.58*	–	13.59*	–	10.68*	–	–	–	–
Brain substance	Experiment	6	1.373	30	1.069	6	0.893	7	0.781	7	0.666	5	0.600	0.334	5.18*
	Control	5	1.330	32	0.837	5	0.640	5	0.600	5	0.600		–	0.199	12.78*
	F		0.07	–	10.90*	–	2.42	–	1.38	–	0.18	–	–	–	–
Leucocytes	Experiment	7	3.357	30	3.282	5	3.494	7	3.673	7	3.618	5	3.160	0.331	2.62*
	Control	5	1.770	19	1.978	4	2.710	5	1.768	5	1.552	–	–	1.015	0.83
	F		10.40*		33.98*	–	2.20	–	16.35*	–	9.30*	–	–		–

The penicillin level is expressed in log (100 × mean of units per ml). The concentration of leucocytes is expressed in log mean number of white leucocytes per ml of cerebrospinal fluid. In the table these expressions are abbreviated to "log".

1) F-values (variance ratio in an analysis of variance) indicated by an asterisk (*) denotes significant differences between means at the level $p < 0.05$

cerebrospinal fluid was low five minutes after the injection After 30 minutes, no penicillin could be definitely found in the cerebrospinal fluid The concurrent concentration penicillin in the blood was higher than in the cerebrospinal fluid

It is important from the clinical point of view to learn how quickly penicillin passes into the cerebrospinal fluid It is probably of greater importance to determine how long penicillin in a therapeutically active concentration remains in cerebrospinal fluid

To increase our understanding of this question, we produced bacterial meningitis in rabbits and thereafter investigated the penicillin concentration in cerebrospinal fluid and brain substance at varying intervals after intravenous administration of benzyl penicillin [1]

MATERIALS AND METHODS

The investigations were conducted on adult rabbits varying in weight between 2 and 3 kg The technique of taking samples and studying the materials was the same *as described in an earlier paper (1) The meningitis was likewise produced in* the experimental rabbits with staphylococcus aureus (strain 209) The dose of penicillin consisted of 20 000 units per kilo The samples in the different series were taken from experimental and control rabbits 15 60 90 and 120 minutes after intravenous injection of penicillin In one series consisting entirely of experimental rabbits samples were taken after 150 minutes Results obtained in an earlier investigation (1) 30 minutes after administration of 20 000 units of penicillin per kilogram of body weight were included for the sake of comparison

RESULTS

Symptoms of meningitis of varying intensity were observed in all the experimental rabbits the day after the experimental infection had been produced The symptoms were classified by severity according to the same system as in the previous paper (1) *i e* +, + + and + + + The distribution of the various symptom pictures in each series was about the same as when the sampling was done 30 minutes after the penicillin injection In the series in which tests were made after 150 minutes, however, the clinical signs at the time of the penicillin injection were in general less intense than in the other series This was due to the fact that rabbits with pronounced signs of meningitis (+ + +) seldom survived long enough, *i e* 150 minutes after the injection

The control animals were completely free of symptoms

The penicillin concentrations in plasma, cerebrospinal fluid and brain substance at different intervals after the injection of penicillin appear from Table 1 and Fig 1 2 and 3, respectively (in Figs 1 2 and 3 broken lines denote controls unbroken lines denote experimental rabbits) Table 1 also shows the concentration of leucocytes in cerebrospinal fluid after the same intervals

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[1] Benzyl penicillin is hereinafter abbreviated to penicillin The penicillin in the present investigation was kindly supplied by AB Kabi Stockholm Sweden

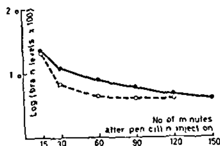


Fig 3

controls was considerably lower at all intervals than in the rabbits with meningitis The difference between experimental and control rabbits was significant after all intervals

There was a relationship between the penicillin concentration in *cerebrospinal fluid and in* plasma both in experimental and in control rabbits The penicillin concentration in cerebrospinal fluid and the intensity of the clinical symptom picture in the experimental rabbits were also related Thus, both in experimental and in control rabbits the ratio $\frac{\text{penicillin concentration in cerebrospinal fluid}}{\text{penicillin concentration in plasma}} \left(\frac{F}{P}\right)$ was higher the later the tests were made after the penicillin injection (Table 2) The level of $\frac{F}{P}$ was clearly higher in experimental than in control rabbits after all intervals

TABLE 2

The ratio $\frac{F}{P}$ in Experimental and Control Rabbits at Different Intervals after the Injection of Penicillin The Quotients are Expressed in per cent

Number of minutes after penicillin injection	% quotient	
	Experiments	Controls
15	8 6	2 0
30	13 1	5 9
60	34 0	18 1
90	39 7	30 9
120	98 9	62 7
150	160 0	–

The concentration of leucocytes (Table 1) increased in experimental rabbits the longer the interval after the penicillin injection up to and including 120 minutes This trend could not be noted in the control rabbits The concentration of leucocytes at almost all intervals was much higher in experimental rabbits than in controls

There was no relation between the concentration of penicillin and of leucocytes in cerebrospinal fluid, except in the experimental rabbits 30 minutes after the penicillin injection

The penicillin concentration in plasma (Table 1, Fig 1) was very high in all the rabbits 15 minutes after the injection of penicillin It had dropped considerably only 30 minutes after the penicillin injection and it continued to decrease in later tests This trend was significant[1] both in experimental rabbits and in controls

At no time was there any significant difference between experimental and control rabbits with regard to the penicillin concentration in plasma

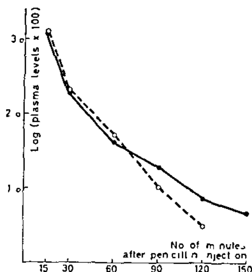


Fig 1

The penicillin concentration in cerebrospinal fluid (Table 1 and Fig 2) was very high in the experimental rabbits 15 minutes after the penicillin injection It had decreased considerably after 30 minutes This downward trend continued with longer intervals and was significant The penicillin concentration in cerebrospinal fluid in the control rabbits also decreased as the intervals between the penicillin injection and the test grew longer The penicillin concentration in the

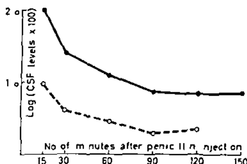


Fig 2

1 Here and in the following this means significant at the level $p < 0.05$

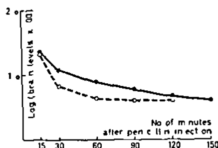


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The concentration of penicillin in brain substance (Table 1, Fig 3) appears to have been somewhat higher in experimental rabbits than in controls at all intervals after the penicillin injection However, the difference was only significant after a 30-minute-interval Both in experimental and in control rabbits, the penicillin concentration in brain substance showed a significant tendency to decrease with increasing intervals after the penicillin injection

DISCUSSION

The time factor is of great importance in the study of the passage of antibiotics into cerebrospinal fluid We want to know how rapidly an antibiotic passes into the cerebrospinal fluid following parenteral administration and also how long it remains there The answers to these questions are of therapeutic significance

In the present experiments, it was found that, both in rabbits with staphylococcic meningitis and in healthy control rabbits, the passage into cerebrospinal fluid was very rapidly following intravenous injection of 20,000 units of penicillin per kg of body weight However, very little penicillin permeated the barrier in control rabbits with intact meninges The concentration in cerebrospinal fluid was very high in experimental animals 15 minutes after the intravenous injection It decreased rapidly in the experimental rabbits at the outset, but was still so high after 150 minutes that it should have some therapeutic value in infections with sensitive bacteria

SUMMARY

1 The passage of penicillin into cerebrospinal fluid and brain substance was studied at varying intervals after intravenous administration of penicillin in rabbits with experimental staphylococcic meningitis

2 The passage into cerebrospinal fluid was very rapid both in experimental and in control rabbits The concentration in cerebrospinal fluid was much higher in the experimental rabbits than in the controls already 15 minutes after the injection of penicillin The concentration in the experimental animals dropped rapidly at first Even two and a half hours later, however, the penicillin remained of some therapeutic value in these animals with bacterial infections in the central nervous system

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THE TREPONEMATOSES DEPARTMENT, STATENS SERUMINSTITUT,
AMAGER BOULEVARD 80, COPENHAGEN S, DENMARK

STUDIES ON THE ANTIGENIC STRUCTURE OF T. PALLIDUM

4. *Comparison between the Cultivable Strains T. Reiter and T. Kazan II, Applying Agar Gel Diffusion Technique and Cross Absorption Experiments*

By

AAGE HEIN CHRISTIANSEN

Received 14.xi.63

As mentioned in a previous paper (4), the Reiter strain of *T. pallidum* maintained in this laboratory was received from *De Bruijn* in Utrecht.

The Kazan strains II, IV, V, and VIII, were kindly sent to Statens Seruminstitut in 1961 by Professor *Ovchinnikov*, Central Research Institute of Dermato-Venereology, Moscow, USSR.

The Kazan strain II was isolated from a papule of a patient with syphilis in 1926 by *Aristofski & Gelzer*. This strain was avirulent for rabbits. Strain V was isolated from the blood of a patient with secondary syphilis in 1940 by *Karimova & Kondratjev*. The third generation was virulent for rabbits, but the virulence disappeared years ago (9). It has not yet been possible to obtain exact information from USSR regarding strains IV and VIII.

The Kazan strains are cultivated in the same manner as the Reiter strain (for details see 4). As regards growth in test tubes and microscopical examination, there is no difference between the Reiter and the Kazan strains.

The question of the identity of the Reiter and Kazan II strains has been studied by *Beck* (1), who found serological identity using absorption and agglutination methods.

Later *Eagle & Germuth* (6) studied the same problem. Unfortunately the number of the Kazan strain used by them is uncertain. On the basis of their cross reactivity in agglutination and complement fixation tests, it was concluded that the Reiter and Kazan strains cross reacted but were not identical.

Dardanoni & Zaffiro (5) found a serological identity between the Reiter strain and the Kazan strain used by *Eagle & Germuth*.

An agar gel diffusion technique is used in the present work in order to reveal a possible difference in the antigenic structure of the Reiter and the Kazan II strains.

MATERIAL AND METHODS

The treponemes were grown in a thioglycollate medium and the antisera were prepared from rabbits by injection of lyophilized treponemes as described previously (4)

Antigens Polysaccharides were prepared according to the principles given in (2) Furthermore an antigenic mixture was made by treatment of a suspension of lyophilized treponemes with ultrasonic waves in the following manner

100 mg of lyophilized treponemes was suspended in 10 ml distilled water and homogenized with a syringe and a coarse needle Then 20 ml of water was added and the suspension poured into a cylindrical brass container closed at the bottom by a 0 2 mm thick plastic membrane with an area of 4 5 cm²

The generator was a Siemens Sonostat Standard of the quartz crystal type with a total intensity of 30 Watt (7) Thus the maximum intensity was 6 7 Watt/cm² when the above mentioned container was placed with the plastic bottom direct on the generator head since the loss of intensity of the ultrasonic waves when penetrating the plastic is negligible The generator head is built in the bottom of a steel jar permitting a continuous flow of tap water for cooling The ultrasonic treatment takes place for 30 minutes After this the integrity of the treponemes is completely lost in most cases Finally the suspension with the liberated antigens is concentrated in vacuum at 45° C to about 5 ml

Technique of Agar Gel Diffusion

The method is a slide microtechnique based on the principles given by *Wadsworth* (10) This method is derived from the original Ouchterlony method (8) the main difference being that the amounts of antigen and antibody are reduced

A gel diffusion chamber of 0 4 mm depth is made by placing two strips of electrical tape on a plexiglass matrix and putting this matrix on a common diapositive slide 5 × 5 cm The matrix was prepared by drilling three holes 3 5 mm i diameter but with a lower aperture of 2 mm in a 35 × 45 mm piece of plexiglass 4 mm thick This provides a basin volume of about 26 µl The distance between the edges of the basins is 2 5 mm The matrix is placed on an agar filtered glass slide and 0 5 per cent melted agar in saline is allowed to flow into the chamber After congelation of the agar the basins are filled with the reactants using constricted pipettes with a capacity of 30 µl Diffusion takes place for 48 hours at room temperature in a humid container and the basins are refilled after the first 24 hours After removal of the matrix the precipitation lines may be drawn photographed dried and coloured with amido black Before drying it is necessary to remove unprecipitated reactants and salts from the agar This may be done by placing the slides in Petri dishes for 24 hours in saline and then substituting the saline by distilled water for another 24 hours

In t … d ultrasonic prepa … immunized with … been reproduced several times Only the most important results are shown in the figures

Besides the agar gel diffusion method the classical cross absorption technique has been applied with immune sera of the two types Thus 2 ml anti Reiter serum was absorbed with 100 mg lyophilized Kazan treponemes for 2 hours at room temperature using continuous shaking The procedure was repeated with anti Kazan serum and Reiter treponemes The sera were tested before and after absorption with the respective polysaccharide antigens in a complement fixation test

RESULTS

The gel diffusion experiments may be divided into three main groups according to the antigens employed *viz* one group using the two polysaccharide antigens, a second using the two ultrasonic prepared antigens, and a third using a polysaccharide and an ultrasonic prepared antigen The main groups may be subdivided according to their antibody type (anti Reiter or anti Kazan)

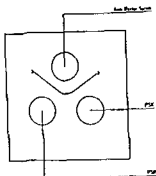


Fig 1

PSK = Polysaccharide from T Kazan
PSR = Polysaccharide from T Reiter

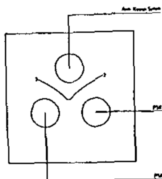


Fig 2

PSK = Polysaccharide from T Kazan
PSR = Polysaccharide from T Reiter

Main group 1 (Polysaccharide Antigens)

Fig 1 shows the precipitation lines using anti-Reiter serum, Reiter polysaccharide (PSR), and Kazan II polysaccharide (PSK) Only one line (named 2) is seen on each side giving a complete fusion in the middle In Fig 2 Kazan II antiserum is tested against the same antigens Here again, two (one on each side) fusing lines are recognized, thus indicating that the two polysaccharides are reacting with the same antibody Thus no serological difference can be demonstrated in the polysaccharide fractions of the two strains

Main group 2 (Ultrasonic Prepared Antigens)

In Figs 3 and 4 anti Reiter or anti-Kazan serum is placed in the top basins, ultrasonic treated Reiter treponemes (USR) in the lower left, and ultrasonic treated Kazan treponemes (USK) in the lower right basin There is no crossing of lines, and thus no serological difference can be demonstrated in this experiment

These experiments reveal three certain antigen-antibody systems common to the two strains The line termed (4) in Fig 4 using anti-Kazan serum is not constant and may be an artefact It appears from the experiments shown in Figs 6–8 that line 2 is the antipolysaccharide line

If, however, the two antisera are mixed (equal parts), a distinct crossing phenomenon is seen, one of the Kazan lines (named 1a) crossing two of the Reiter lines (Fig 5) This reveals at least one antigen-antibody system related to the Kazan strain and not found in the Reiter strain

Main group 3 (Ultrasonic Prepared and Polysaccharide Antigens)

The ultrasonic treated Reiter treponemes contain at least three different antigens In order to see which of them was the polysaccharide

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In the following experiments all combinations with polysaccharide and ultrasonic prepared antigens have been examined against antisera from rabbits immunized with lyophilized Reiter or Kazan treponemes All the experiments have been reproduced several times Only the most important results are shown in the figures

Besides the agar gel diffusion method the classical cross absorption technique has been applied with immune sera of the two types Thus 2 ml anti Reiter serum was absorbed with 100 mg lyophilized Kazan treponemes for 2 hours at room temperature using continuous shaking The procedure was repeated with anti Kazan serum and Reiter treponemes The sera were tested before and after absorption with the respective polysaccharide antigens in a complement fixation test

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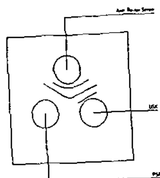


Fig 7

USK = Ultrasonic prepared antigens from T Kazan
PSR = Polysaccharide from T Reiter

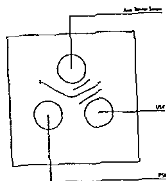


Fig 8

USK = Ultrasonic prepared antigens from T Kazan
PSK = Polysaccharide from T Kazan

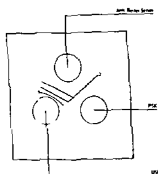


Fig 9

PSK = Polysaccharide from T Kazan
USR = Ultrasonic prepared antigens from T Reiter

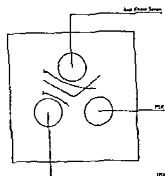


Fig 10

PSK — Polysaccharide from T Kazan
USK = Ultrasonic prepared antigens from T Kazan

crossing line indicates the existence of a serological system quite different from the polysaccharide system and specific for the Kazan strain

When the Kazan antiserum is tested against the various antigens from the two strains a spur formation is seen in the polysaccharide system (Figs 11 13 line 2) The spur appears in any combination of Reiter and Kazan antigens and even if the Reiter antigens are used alone This phenomenon suggests a complexity in the Kazan serological system not recognized in the corresponding Reiter system

Cross Absorption Experiments

Table 1 shows the results of complement fixation tests applied to Reiter and Kazan antisera before and after absorption with the treponemes The respective polysaccharides have been used as antigens By cross absorption and using the polysaccharides as antigens in the

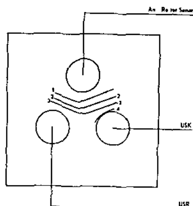


Fig 3

USK = Ultrasonic prepared antigens from T Kazan
USR = Ultrasonic prepared antigens from T Reiter

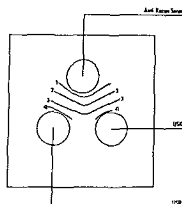


Fig 4

USK = Ultrasonic prepared antigens from T Kazan
USR = Ultrasonic prepared antigens from T Reiter

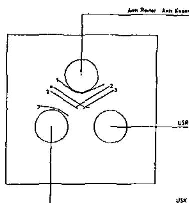


Fig 5

USK = Ultrasonic prepared antigens from T Kazan
USR = Ultrasonic prepared antigens from T Reiter

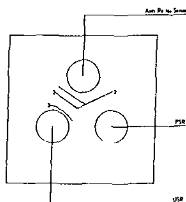


Fig 6

PSR = Polysaccharide from T Reiter

USR = Ultrasonic prepared antigens from T Reiter

component, a Reiter antiserum was tested against USR and PSR (Fig 6) The fusing lines (named 2) indicate the polysaccharide component A repeated experiment with USK shows a similar pattern (Fig 7)

When the anti Reiter serum is tested against ultrasonic prepared Kazan antigens, and Kazan and Reiter polysaccharides are used as references respectively, it is confirmed that line 2 represents the polysaccharide system These experiments also confirm the serological identity of the polysaccharides (Figs 7 and 8) When anti-Reiter serum is tested against USR and PSK as references, the position of the polysaccharide line is confirmed again (Fig 9)

Testing of Kazan antiserum against USK and PSK (Fig 10) gives a fusion of the lines named 2 and a crossing with another (1a) The

morphology of the colonies on the plates This observation is in accordance with the difference between the two strains revealed by applying the gel diffusion technique

DISCUSSION

The cross absorption experiments and the complement fixation test reveal no qualitative serological difference between the purified polysaccharides of the two strains of treponemes This is in agreement with the results from the agar gel diffusion using the same antigens (Figs 1 and 2) The conclusion must be that the purified polysaccharides of the two strains are serologically identical

Nor does testing of the antigen mixture after ultrasonic treatment of the two strains against the respective antisera reveal any certain serological difference If however the antisera are mixed and tested against the ultrasonic prepared antigens one of the Kazan lines crosses two of the Reiter lines thus indicating a serological system specific for the Kazan strain (Fig 5) and different from the polysaccharide system (Fig 10)

Comparison of the slides in Main Group 3 (Figs 6–13) shows that ultrasonic prepared Reiter and Kazan antigens reveal three serologic systems both in the Kazan and Reiter antiserum

The spur formation (Figs 11 12 13) suggests a complexity of the polysaccharide system since in addition to the reaction with the purified polysaccharide antigen the antipolysaccharide antibody is reacting with a possibly more specific component in the antigen of polysaccharide nature found in the ultrasonic prepared antigens

It seems reasonable to postulate that the Kazan strain contains at least one antigen completely different from the Reiter strain because of the above mentioned crossing precipitation line termed 1a (Fig 5) This line is different from the polysaccharide system (Fig 10)

The lack of crossing lines (Figs 3 and 4) when ultrasonic prepared antigens of the two strains are tested against the respective antisera while the crossing of one Kazan line with two of the Reiter lines is evident when the two types of antisera are mixed in one basin (Fig 5) reveals the possibility of the existence of an antibody in the Kazan immune serum induced by an antigen specific for the Kazan strain (in comparison with the Reiter strain)

The present experiments indicate that the Reiter and Kazan strains contain a common polysaccharide antigen reacting with a homologous antibody in both antisera Furthermore two other antigen antibody systems have been demonstrated which seem to be common to the two strains Finally an antigen antibody system was found in the Kazan strain which is not demonstrated in the Reiter strain

As regards comparison of the antigenic structure of the Reiter and the Kazan II strain of treponemes it may therefore be reasonable to

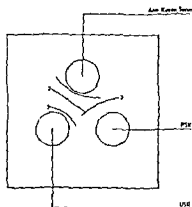


Fig 11
PSK = Polysaccharide from T Kazan
USR = Ultrasonic prepared antigens from T Reiter

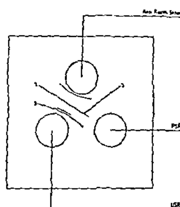


Fig 12
PSR = Polysaccharide from T Reiter
USR = Ultrasonic prepared antigens from T Reiter

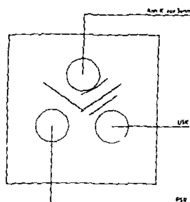


Fig 13
USK = Ultrasonic prepared antigens from T Kazan
PSR = Polysaccharide from T Reiter

TABLE 1

Complement Fixation Test Employing Kazan II and Reiter Polysaccharides before and after Cross Absorption of Antisera with the Respective Treponemes*

Antiserum	Before absorption		After absorption	
	Kazan polysaccharide	Reiter polysaccharide	Kazan polysaccharide	Reiter polysaccharide
Kazan	11	12	—	
Reiter	9	11		

* In degrees of strength

test, no serological difference could be demonstrated This was to be expected, judging by the result of the experiments in Main Group I

The Reiter and Kazan strains have recently been cultivated on plates in this laboratory It was interesting to find a marked difference in the

FROM THE HOSPITAL FOR INFECTIOUS DISEASES AND THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF UPPSALA, SWEDEN

AN EXTENSIVE OUTBREAK OF GASTROENTERITIS CAUSED BY SALMONELLA NEWPORT

3. *Serum Agglutinins in 412 Hospitalized Cases*

By

Folke Nordbring, Bo Bille and Tore Mellbin

Received 21.xi.63

An extensive outbreak of food poisoning due to *Salmonella Newport* occurred in Uppsala at the end of April and the beginning of May 1960. The source of infection was contaminated butter cream in cakes from a large bakery. About 750 known cases appeared in the Uppsala region, including the carriers.

Detailed reports on this outbreak have been given in previous articles (2, 21). From these it appears that approximately 35 per cent of the known patients developed symptoms, while the remaining 65 per cent were carriers only. The symptoms were mainly those of acute gastroenteritis with fever, diarrhea, abdominal cramps and vomiting as the predominating signs. It is, however, striking that a high proportion of the patients with clinical illness had severe symptoms with high fever and prostration, although only a few appeared critically ill. Septicaemia was apparently common, and positive blood cultures were obtained in about 60 per cent of the cases analysed in this respect. There was evidence of a heavy contamination of the foodstuff responsible for the outbreak, and the ingestion of a large dose of bacteria could thus be presupposed.

It has been pointed out (13, 27) that practically every salmonella type that gives rise to the gastroenteritis syndrome is capable of producing a generalized disease. *Saphra & Winter* (23) are of the opinion that a transient bacteraemia is probably the rule rather than the exception in salmonella infections with fever. Although infection with *S. Newport* mostly gives rise to the gastroenteric type of salmonellosis, it is evident from this outbreak that invasion of the blood

conclude that, although closely related, they are not completely identical

This conclusion is emphasized by the above mentioned observation of a pronounced difference in the morphology of the colonies of these strains when cultivated on plates

It is interesting that the purified polysaccharide does not seem to be the most specific fraction in closely related treponemal strains The nature of the above mentioned antigen of the Kazan II strain, which is possibly specific for that strain, is still uncertain

It may perhaps be profitable to focus attention on the nucleoprotein fractions, at least as regards closely related strains

SUMMARY

Cross absorption experiments and gel diffusion technique have shown that the purified polysaccharides from Reiter and Kazan II strains of treponemes isolated by the phenol water method are serologically indistinguishable

In addition to the polysaccharide system, three other serological systems have been revealed by ultrasonic treatment of these treponemes At least one of these seems to be specific for the Kazan II strain when compared with the Reiter strain

Thus the Kazan II and the Reiter strains are closely related but not identical in the antigenic structure

It is suggested that the polysaccharide is not the most specific component in closely related strains of treponemes, but other fractions (possibly nucleoprotein) must be borne in mind

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stances only one sample was obtained in 25 instances three samples. With very few exceptions at least one sample was secured *not earlier than 14 days after the onset* of the illness. In cases where only the day of contamination was known at least one sample was drawn not earlier than 17 days afterwards and in cases with no illness and unknown day of contamination at least one sample was obtained not earlier than 20 days after the middle of the actual period of contamination (May 4th). The serum was separated and kept frozen until analysis. All sera from a patient were analysed at the same time and the highest titre found in one of the two or three samples was counted. Altogether, 790 sera were available for analysis.

TABLE 1

Grades of Illness among the Patients Admitted and the Patients Examined for Antibodies

Symptoms	Patients admitted N = 498 %	Patients investigated N = 412 %
Severe	1.8	1.5
Moderately severe	27.5	27.4
Slight	9.6	9.7
Carriers	61.1	61.4

Method.—O and H agglutinin titres were determined by the serial dilution test (Widal test). The following antigens were used O VIII eh (phase 1) and 1.2 (phase 2) all prepared in the State Bacteriologic Laboratory, Stockholm. All readings were [illegible] was positive.

RESULTS

The results will be presented in diagrams and tables.

Fig. 1 shows the distribution of the O VIII-agglutinin titres among three categories of patients with different clinical features: those with severe or moderately severe symptoms (A), those with mild symptoms (B), and those [illegible]
in faeces (C [illegible]

[illegible] is further demonstrated in Table 2. O agglutinin titres of 320 or higher are found in 100 per cent of the sera of the severely ill, in 92.9 per cent of the patients with moderately severe symptoms, in 52.5 per cent of the slightly ill, and in 40.7 per cent of the carriers. Positive O-agglutinin titres are recorded in 57 per cent of the total material.

The distribution of the H agglutinin titres among the same groups of patients is quite different from the foregoing, as is seen from Fig. 2, Fig. 3 and Table 2. Titres of eh agglutinins at the level of 320 or higher are found in 21.4 per cent of the total material, and corresponding titres of 1.2 agglutinins in 18.4 per cent. The great majority of the carriers have no determinable H agglutinins, and in only about four

The patients in the present epidemic were isolated either at home or in hospitals. 488 patients were admitted to the Hospital for Infectious Diseases in Uppsala. These patients exhibited various degrees of illness or were carriers. An opportunity to study the development of antibodies against *S. Newport* in different phases of disease was thus offered. Of particular importance in this study were the facts that practically none had been immunized with salmonella vaccine previously, and very few had received treatment with chloramphenicol during the course of the disease.

There is a great deal of data in the literature concerning the use of the serum dilution test for agglutinins in the diagnosis of typhoid and paratyphoid fevers. Similar studies in connexion with the salmonella gastroenteritis syndrome are much more sparse. The general conception is that the Widal test is of very limited value in cases of food poisoning due to salmonellae (29). *Bornstein* (3, 4), however, states that agglutinins appear in the serum also in the majority of cases of gastroenteritis, contrary to the former belief. In surveys of infections with *S. typhimurium Kristensen et al.* (18) and *Bengtsson et al.* (1) found positive Widal tests in 86 and 89 per cent, respectively.

Seligman et al. (27) found a positive serologic response in salmonella infections with generalized symptoms or with severe or prolonged manifestations of gastroenteritis. *Eisenberg et al.* (8), however, gained the impression that there was no correlation between the magnitude of antibody titre and type of clinical syndrome or degree of illness, but their material was small.

There are isolated reports on the appearance of agglutinins to *S. Newport* in the literature (11, 20), but no major study on infections with this type of salmonella has been found. On the whole, there is a lack of investigations on the agglutinin response in cases of gastroenteritis, as has been pointed out by investigators well acquainted with the subject (24).

MATERIAL AND METHODS

Clinical material.—Blood samples for agglutinin determination were obtained from 412 patients out of the 488 admitted (84.4 per cent). 212 patients were females and 200 males. Adult patients predominated. 312 (75.7 per cent) were above 16 years of age, 95 (23.1 per cent) were 7–15 years old, and 5 (1.2 per cent) were from four to six years old.

The patients admitted were classified in various grades of illness, a classification which has been discussed in more detail in previous papers. Briefly, patients with *severe* symptoms exhibited fever, diarrhea and/or vomiting, disturbed fluid balance, sensory alterations and often a markedly septic state; patients with *moderately severe* symptoms had fever above 39° C, diarrhea and/or vomiting, but their condition was not serious; patients with *slight* symptoms presented diarrhea and/or vomiting but had no fever; the *carriers* developed no symptoms at all. The distribution of the material investigated among the grades of illness is seen from Table 1.

One patient only had been immunized with typhoid-paratyphoid vaccine within the last two years. Three patients had been vaccinated several years previously.

Blood samples.—In most instances two blood samples were drawn from each patient, the first on admission, the second usually about 10 days later. In 73 in-

stances only one sample was obtained in 25 instances three samples. With very few exceptions at least one sample was secured not earlier than 14 days after the onset of the illness. In cases where only the day of contamination was known at least one sample was drawn not earlier than 17 days afterwards and in cases with no illness and unknown day of contamination at least one sample was obtained not earlier than 20 days after the middle of the actual period of contamination (May 4th). The serum was separated and kept frozen until analysis. All sera from a patient were analysed at the same time and the highest titre found in one of the two or three samples was counted. Altogether 790 sera were available for analysis.

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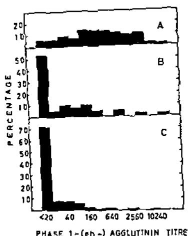


Fig 2

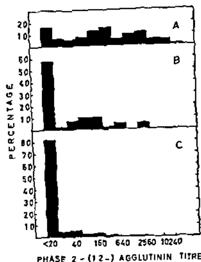


Fig 3

Fig 2 The distribution of eh agglutinin titres in the same categories of patients as in Fig 1

Fig 3 The distribution of 1 2-agglutinin titres in the same categories of patients as in Fig 1 and Fig 2

Very high titres, of 10,240 or higher, were found in 15 instances (3 6 per cent) An O VIII-agglutinin titre of this magnitude was noted once, eh agglutinins seven times and 1,2 agglutinins also seven times The highest H-agglutinin titre recorded was 20,480

The correlation between O- and H-agglutinin titres is presented in Figs 4 and 5, in which O titres are plotted against H titres These figures show many cases with a positive O-agglutinin titre but with a negative H-agglutinin titre However, almost every serum with a positive eh- or 1,2 agglutinin titre displays a coexisting, positive O-agglutinin titre This occurs in 96 6 per cent of the eh-positive sera and in 94 7 per cent of the 1,2-positive sera Only three or four sera with positive H-agglutinin titres show negative O-agglutinin titres

Fig 6 illustrates the presence of eh agglutinins and 1,2 agglutinins in the sera analysed A positive titre of eh agglutinins is found in 8 5 per cent of the sera, of 1,2 agglutinins in 5 5 per cent, and of both H agglutinins in 12 9 per cent

Appearance of positive H-agglutinin titres is almost invariably combined with positive O agglutinin titres, as has already been pointed out In fact the data obtained reveal that a complete serologic response to the three antigens used or a response to the O antigen in combination with *one* H antigen occurs almost exclusively in patients who are ill This is demonstrated in Table 3 A total agglutinin response is observed in 37 per cent of the patients with severe or moderate symptoms but only in 1 6 per cent of the carriers A development of O agglutinins only

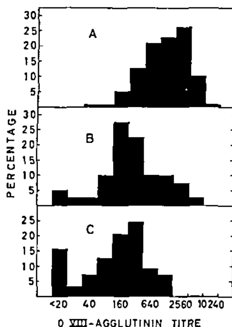


Fig 1

The distribution of O VIII-agglutinin titres in three defined categories of patients A denotes patients with severe or moderate symptoms B patients with mild symptoms, and C carriers with no symptoms

per cent are titres found above the level of 320 The titre levels of H agglutinins are evenly distributed among the patients with severe or moderately severe symptoms, and, as before, the patients with slight symptoms have a titre distribution which is something between the extremes

TABLE 2

The Percentage Distribution of Agglutinin Titres among Patients with Different Clinical Features

Symptoms	No of patients examined	Titre 320 or higher			Titre 2560 or higher		
		O VIII %	eh %	1 2 %	O VIII %	eh %	1 2 %
Severe	6	(100.0)	(83.3)	(83.3)	(66.7)	(66.7)	(66.7)
Moderately severe	113	92.9	59.3	50.4	35.4	21.2	19.5
Slight	40	52.5	15.0	12.5	10.0	5.0	5.0
Carriers	253	40.7	4.0	3.6	0	1.6	0.4
Total	412	57.0	21.4	18.4	11.7	8.3	7.0

The correlation between serologic response and grade of illness is also obvious at a higher titre level, as is seen from Table 2 Throughout, cases with an agglutinin titre of 2560 or higher are most common among the markedly ill patients Only very few carriers have H-agglutinin titres above 2560, and no carrier displays an O-agglutinin titre of this high level

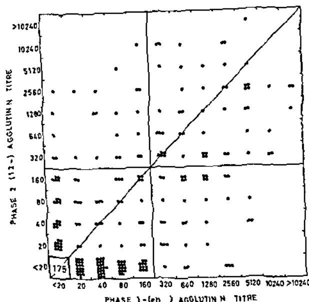


Fig 6

The correlation between ch and 1 2 agglutinin titres in the same material as in Fig 4 and Fig 5

is more common among the carriers and the slightly ill than among those with more severe symptoms, and no response at all is a characteristic feature among the carriers

A rise in titre of three steps or more as regarded O agglutinins, which might be considered significant (22), was noticed only in two of 336 paired sera (0 6 per cent), as regarded ch agglutinins in seven instances (2 1 per cent) and as regarded 1,2 agglutinins in 12 instances (3 6 per cent) A fall in titre of the same magnitude of the corresponding agglutinins was noticed in 1 2, 0 9 and 1 2 per cent, respectively

TABLE 3

Response to Different Antigens in Groups of Patients with Varying Symptoms

Titre 320 or higher against	Symptoms		
	Severe or moderately severe N=119 %	Slight N=40 %	Carriers N=233 %
O VIII + ch + 1,2	37 0	10 0	1 6
O VIII + ch	21 0	5 0	2 3
O VIII + 1 2	12 6	2 5	1.2
O VIII	22 7	35 0	35 9
ch + 1 2	0 8	–	–
ch	1 7	–	
1,2	0 8	–	0 8
None	3 4	47 5	58 1

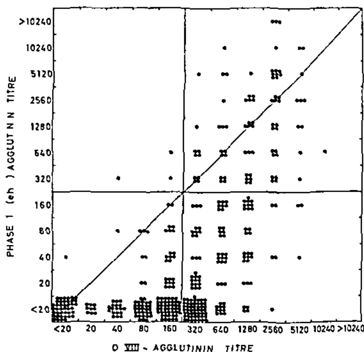


Fig 4

The correlation between O VIII and eh agglutinin titres in 412 patients The horizontal and vertical lines indicate the chosen borders between negative and positive titres

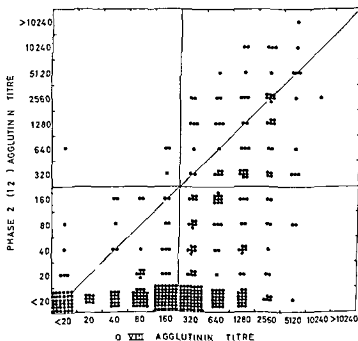


Fig 5

The correlation between O VIII and 1 2 agglutinin titres in the same material as in Fig 4

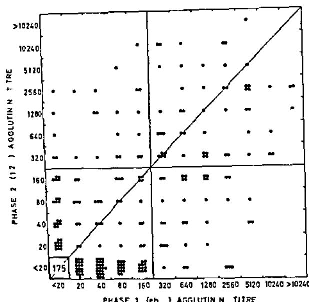


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eh + 1.2	0 8		
eh	1 7	–	
1.2	0 8		0 8
None	3 4	47 5	58 1

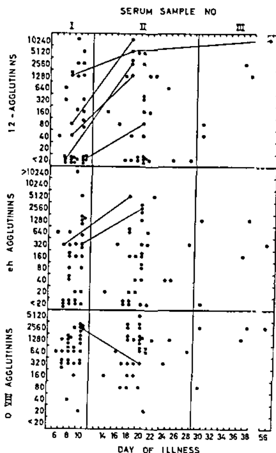


Fig 7

The agglutinin titres of the first and second sera in 32 cases and of the third serum sample in 5 cases The lines indicate a change in titre of three steps or more

In a small group of cases (32 patients), the first serum was taken on the sixth to tenth day of illness The titre levels of these sera are shown in Fig 7, which also presents the titres of the second and third sera from these patients A rise in 1,2 agglutinins of three steps or more is noticed five times, in eh agglutinins twice, but in O agglutinins in no case

Fig 8 gives another illustration of the development of the antibodies analysed The mean measurable titre of the various agglutinins obtained in each of the first four weeks are plotted It is seen that the courses of the curves for O agglutinins and eh agglutinins follow each other closely, although on different levels, with the maximum attained in the second week, while the highest mean level of 1 2 agglutinins is reached in the third week The course of events in the following two years is at present being studied in a group of patients and will be reported later

An attempt was made to compare the antibody production in children and in adults Because of the proportionately small number of sera available from children with symptoms, sufficient data for the calcula-

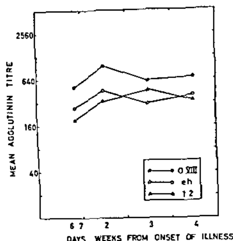


Fig 8

The mean measurable O and H agglutination titres of the first four weeks of illness

tion of weekly, mean antibody titres could be obtained only during the second and the fourth weeks of illness The mean values of measurable titres (20 or higher) are presented in Fig 9 The values are computed from a material of 24 sera from 14 children aged 4–15 years (mean age 8 8 years) and from a material of 122 sera from 85 adult persons aged 16 81 years (mean age 41 9 years) All children had a disease of moderate severity according to the definitions 93 per cent of the adult patients had a disease of corresponding severity and seven per cent were

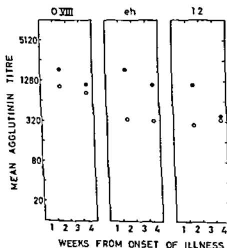


Fig 9

The mean measurable O and H agglutinin titres of the second and fourth weeks of illness in children and adults ● denotes children ○ denotes adults

seriously ill All sera from the children had measurable O VIII- and eh agglutinin titres, while the 1,2-agglutinin titres were below 20 in 16 7 and 8 3 per cent of the samples in the two weeks investigated All sera from the adult patients had also measurable O VIII titres, but titres of eh agglutinins were below 20 in 8 3 and 4 0 per cent, and of 1,2 agglutinins in 23 6 and 14 0 per cent All patients in the two age groups knew exactly the day of onset of the illness

Although the severity of the disease was somewhat more pronounced among the adult patients, it is striking that the average titre of the various antibodies is higher among the children throughout, at times considerably higher A similar tendency, though less prominent, was found among the carriers in the different age groups

DISCUSSION

There is strong evidence from the clinical observations (21) that the infection with *S Newport* in the present outbreak in many instances resulted in invasion of the blood stream by the bacteria from the gastrointestinal tract The results of the present investigation show very definitely a correlation between the agglutinin response and the severity of the disease This is true for all three types of antibodies Thus, the highest titres of O and H agglutinins are found among patients with severe or moderately severe symptoms Further, a combined response to both O and H antigens is typical of the patient with marked illness but seldom occurs among patients with mild illness or no illness at all By using different antigens in the test, it was thus possible to score the patients with regard to severity of infection on the basis of formation of agglutinins It should again be pointed out that the two procedures of grouping the patients according to the various degrees of illness and analysing the sera on antibodies were independent of each other

A combined response to the O VIII antigen and to one or two of the H antigens used was noted in 84 of 119 patients with severe or moderately severe symptoms (70 6 per cent) Such a response occurred in 13 of 253 carriers (5 1 per cent) A closer study of the records of the 13 carriers who developed agglutinins of at least two types reveals that one person was immunized with typhoid paratyphoid vaccine *shortly* before admission to hospital, and four patients had fever and mild gastrointestinal disturbances some days after eating the contaminated food These signs were combined with some symptoms of upper respiratory infection, and therefore these patients were regarded as having doubtful signs of salmonellosis and were classified as carriers Retrospectively, these four 'carriers' should probably be considered as patients with clinical illness Eight carriers remained who had not had any symptoms at all but nevertheless developed two or three types of *agglutinins* This means that a combined serologic response was revealed only in 3 2 per cent of the carriers Five of these eight carriers

were younger than 16 years of age. Slight symptoms of illness might have been overlooked by the patients or their parents or were not mentioned for other reasons. The predominance of children might be a manifestation of the tendency to a more marked agglutinin response in children which has been presented above.

The high percentage of sera from patients with clinical illness showing an antibody response against one or more antigens may be interpreted as a sign of the invasive property of the infecting organism. Certainly some differences between one salmonella species and another do exist with regard to pathogenicity and serologic response, as is particularly evident from the experiments of *McCullough & Eisele* (19), and as has been discussed more fully by *Hook* (16) and *Gezon* (13).

A fair proportion of the sera had high titres of agglutinins. All titres above the level of 2560 were found in patients with symptoms, except in one or two cases. About one fifth of the 159 patients with clinical illness had an H-agglutinin titre of 2560 or above, and about one-third had an O agglutinin titre of this magnitude. Although the great majority of the very high titres (above 5120) were H agglutinins, it is surprising to find such a large number of high O agglutinin titres as mentioned above, particularly when the results are compared with others in the literature (6, 27). However, the titre level recorded is highly dependent on the technique of the readings, and careful studies have revealed that a comparison of the test results obtained in different laboratories is futile (12).

The fact that only one or two patients were treated with chloramphenicol prior to the antibody analysis may be of importance. It has been pointed out by many investigators (9, 14, 17, 26, 28) that the administration of chloramphenicol depresses or delays the agglutinin response in typhoid fever, at any rate as regards the O agglutinins. Some authors (14, 17) used to find considerably higher titres in the same laboratories in previous years.

The maximum titre level in individual cases was often reached as early as at the end of the first week or at the beginning of the second week. The highest mean level of O agglutinins and phase 1 agglutinins occurred in the second week, while the maximum level of phase 2 agglutinins appeared one week later. The difference observed between the two types of H agglutinins may possibly be due to phase variation in a number of instances with a transformation from phase 1 to phase 2 flagellar antigens during the course of the disease.

The requirement of two serum samples with rising antibody titre from the first to the second one for serologic diagnosis of any infectious disease is generally accepted and rightly so. It is, however, not always possible to fulfil this requirement, because the patient is not always seen until many days have passed and the first "acute" [illegible]
[illegible]

paired sera without three- or fourfold difference in titre as a serologic corroboration of a bacteriologic diagnosis, provided that the patient has not been immunized recently and has not received broad-spectrum antibiotics early in the course of the illness, and that the actual type of salmonella infection is not endemic in the area The titre level regarded as positive will depend on the technique in the local laboratory, an O agglutinin level of 160 often being considered as diagnostic (7, 10) The presence of both O and H agglutinins appears much more conclusive

SUMMARY

The development of serum agglutinins against *Salmonella Newport* has been studied in 412 patients, hospitalized because of infection with this type of salmonella The patients exhibited symptoms of varying severity or were carriers Agglutinins against three antigens (O VIII, eh and 1,2) were determined

A striking correlation between deegree of illness and serologic response was revealed Thus, the percentage of sera with high titres was highest among the severely ill, decreased gradually among the groups of patients with milder symptoms and was lowest among the carriers This was true for all three types of agglutinins analysed

A combined response to the O VIII antigen and to one or two of the H antigens was noted in 70 6 per cent of the patients with severe or moderately severe symptoms but only in 3 2 per cent of the carriers

The maximum titre level was most often found as early as at the end of the first or beginning of the second week, and a significant rise in titre in paired sera was seldom observed, because the first serum was usually not obtained until several days after the onset of the illness, at a time when antibodies were already present It is pointed out that this must often be the case in clinical work, but antibody analysis may nevertheless be of value in cases of salmonella gastroenteritis

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FROM THE UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY (HEAD O TORGERSEN M D) AND THE MEDICAL DEPARTMENT B (HEAD CHR BJERKELUND M D) RIKSHOSPITALET OSLO NORWAY

REGENERATION OF PARIETAL CELLS FOLLOWING EXPERIMENTALLY PRODUCED GASTRIC ULCERS IN MICE

Intracellular Succinic Dehydrogenase Activity and Gastric pH in Untreated Animals

By

J MYREN and O TORGERSEN

Received 8 vii 63

Epithelial regeneration of the gastric mucosa following experimentally produced ulcers has been extensively studied by various techniques in many types of animals (*Ferguson* 1928 *Gunther* 1950, *Williams* 1953 54 *Myhre* 1959 *Messier* 1960 *Torgersen* 1961 and *Townsend* 1961)

These authors agree that new undifferentiated epithelium quickly regenerates from the surrounding mucosa and in a few days gland like structures are formed

On the other hand little is known about the possible regeneration and differentiation of the more specialized cells of the gastric mucosa This applies as well to the morphological as to the functional aspects of these questions

Concerning the parietal cells it is generally believed that these do regenerate following experimentally produced lesions Thus parietal cells have been observed in the regenerated area from the 20th day and onwards (*Ferguson* 1928 *Williams* 1953 54 *Finckh & Milton* 1960 *Townsend* 1961) In these experiments however lesions of varying size have been produced by mechanical methods and the rate of regeneration has been difficult to evaluate

The functional aspects of the parietal cells in stomachs s[illegible] ted to sugg[illegible] [illegible] d Un de[illegible] (SD) ac[illegible] of the g[illegible] *y et & Willighagen* 1960) indicating a particularly high metabolic activity in terms of the energyyielding processes of the Krebs cyclus In humans careful registrations in this way have demonstrated a significant correlation between the amount of acid secreted by the stomach and the number of parietal cells present in

BRIEF REPORT

SPLENIC EOSINOPOIESIS IN THE NORMAL RAT

By *Frede Bro Rasmussen* and *O Henriksen*

The evaluation of the spleen as the site of formation of eosinophil leukocytes in man differs within quite wide limits but generally eosinopoiesis in the spleen is considered a pathological phenomenon

Animal experiments too have given varying results

In the effort to elucidate the possibility of eosinopoiesis in the spleen we studied rat spleens (35 experimental animals) using absolute counts (modified Dunger method 1910 *Andersen* 1943) of the eosinophil cells obtained by shaking spleen homogenizates smears of the homogenizates and stained sections

It is our impression that the risk of mechanical destruction of the eosinophil leukocytes is greatly exaggerated

The experimental animals were female rats (Leo) weighing about 150 g Before the experiments were started the rats were acclimatized to the Institute after having been isolated one to a cage During the period of acclimatization blood specimens were obtained by amputation of the tip of the tail for determination of haemoglobin content differential count absolute count of nucleated blood cells and eosinophil granulocytes The main investigations comprising apart from the spleen also the bone marrow and gastric mucosa were supplemented by studies of the cardiac or aortic blood Unlike previous authors (cf *Studer* 1950) we did not find a significant difference between the eosinophil count in tail blood and cardiac or aortic blood provided that the tail blood could flow freely while the specimen was being drawn

In all cases eosinophil granulocytes were found in the specimens of the spleens A perifollicular localization of the eosinophil leukocytes in major or minor clusters was a characteristic and constant finding Eosinophil cells were never found within the follicles

The ultimate maturation stages of neutrophil granulocytes in the rat consist in an alteration of the nuclear morphology From being large irregular and immature the nuclei change to smaller notched annular and further to segmented nuclei The maturation of the eosinophil granulocytes runs through the same stages but never seems to get beyond the stage of the annular nuclei in some cases twisted into a figure of eight or pretzel shape

In the above mentioned clusters of eosinophils in sections of the spleen there were eosinophil cells in all stages of maturation However cells with annular nuclei were extremely sparse outside vessels and sinusoids in which they were present in association with and in the same proportion as blood elements in the peripheral blood Definite mitotic figures were not observed in the eosinophil cells in any case

Evaluation of the nuclear morphology of the eosinophil cells is difficult to perform in sections better in smears of cells obtained by shaking spleen homogenizates this does not appear to have been done previously Smears of tissue from the bone marrow and splenic tissue show a comparable distribution of the preliminary stages of eosinophil leukocytes although the spleen contains a relatively smaller number than the bone marrow

We interpret the results as evidence of splenic eosinopoiesis in the normal rat We did not observe a definite parallelism between the eosinophil count in the peripheral blood and in the spleen and splenectomy did not result in a significant decrease in the absolute and relative number of eosinophil leukocytes in the peripheral blood It cannt be ruled out however that this may have been due to an uncertainty of the method of counting (differential counts and absolute counts of leukocytes and eosinophil cells)

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ocular diaphragm with 0 3 mm interval in the full length within the sections excluding the ulcer defect (Fig 1) In each section also the longitudinal extension of the layer containing SD active parietal cells was determined including the injured area (Fig 1)

RESULTS

In control mice the average thickness of the mucosal layer containing SD active parietal cells was 0 2 mm (standard deviation $\left(s = \sqrt{\frac{\Sigma(x^2)}{n}} = \right.$ 0 06), and the average longitudinal extension of this layer was 3 4 mm (s 1 4) (Table 2) In these animals the average value of the pH in the gastric contents near to the mucosal surface was 2 2 (s 0 34), and the degree of succinic dehydrogenase activity in the parietal cells was + + in 11 and + in 2 mice (Table 1)

TABLE 1

Average Values of pH of Gastric Content at the Surface of Mucosa and of Succinic Dehydrogenase Activity of Parietal Cells in 112 Mice

	pH	s	Degree of SD activity			No of mice
			+	++	+++	
Controls	2 2	0 34	2	11		13
After operation						
1 hour	5 2	0 76	10			10
3 hours	4 0	0 86	1	9		10
24 „	2 7	0 56	1	4	1	6
3 days	2 4	0 26	2	7		9
5 „	2 3	0 62	3	7		10
7 „	2 8	1 02	1	5		6
2 weeks	2 9	1 00	3	14	2	19
4 „	2 4	0 58	2	12	1	15
13 „	2 2	0 36	3	5		8
25	2 1	0 14	1	6		7

TABLE 2

Size (Length) of Local Defect in Parietal Cell Layer at Various Stages Following Healing Thickness (Depth) and Length (Total Longitudinal Extension) of Parietal Cell Layer 112 Mice

	Layer containing parietal cells mm						
	Local defect	s	Thickness	s	Length	s	No of mice
Controls			0 20	0 08	3 4	1 4	13
1 hour	0 15	0 14	0 16	0 06	4 4	1 2	10
3 hours	0 17	0 14	0 19	0 06	3 1	1 2	10
24	0 32	0 30	0 17	0 08	2 9	1 4	6
3 days	0 98	0 26	0 08	0 02	2 7	0 4	9
5 „	0 90	0 32	0 08	0 02	3 0	0 6	10
7 „	1 10	0 50	0 08	0 06	2 9	1 6	6
2 weeks	0 93	0 48	0 11	0 06	2 5	1 2	19
4	0 60	0 36	0 13	0 08	3 0	1 2	15
13 „	0 84	0.36	0 07	0 06	2.9	1 4	8
23 „	0 70	0 14	0 09	0 04	2 7	0 8	7

the gastric mucosa estimated as well by ordinary histological methods (*Card & Marks* 1960, *Myren et al* 1962) as by the SD-activity of the parietal cells (*Myren et al* 1962)

The following problems are the subject of the present paper Firstly, does regeneration of parietal cells occur in an ulcer area where all epithelial cells have been completely destroyed? Secondly, does a local damage to the gastric wall lead to any morphological or functional alteration, in terms of intracellular SD-activity and gastric pH, of parietal cells situated outside the injured area? If so, does regeneration or restitution occur of these cells?

MATERIAL AND METHODS

White male and female mice 4 to 10 months old, were used Following laparotomia in ether narcosis a small copper rod (diameter 2 mm), kept at a constant temperature of 55° C was pressed gently against the anterior peritoneal surface of the gastric fundus for 15 seconds (*Torgersen* 1961) This resulted in local necrosis and ulceration of the corresponding gastric mucosa with subsequent regeneration of non differentiated epithelium a few days later (Fig 1) The mortality of the operation procedure was about 20 per cent

Out of the total number of 112 mice 13 served as controls the others being examined after one hour to 25 weeks (Tables 1 2)

After sacrification of the animals by neck fracture the stomach was immediately removed opened along the greater curvature and stretched on a cork plate The pH of the gastric contents was determined near to the mucosal surface by a pH paper graded from 1 to 10 After removal of the gastric contents a pin was placed cranially to the border of the ulcer region which was usually distinctly visible to the naked eye The gastric mucosa was then gently frozen with ice of CO_2 and cut with a razor blade longitudinally close to the pin the ulcer area thus being divided into two equal parts One of these was transferred to 10 per cent formalin and embedded in paraffin the other was further frozen on ice of CO_2 and cryostat sections 6 to 7 microns thick were made of the full length of the gastric wall through the ulcer area parallel to the cut margin At least two sections were examined for succinic dehydrogenase by the method of *Pearse* (1960) with MTT (3 (4 5 dimethylthiazolyl 2) 2 5 diphenyl tetrazolium bromide)

The degree of succinic dehydrogenase activity of parietal cells was evaluated at + ++ and +++ according to the amount of black granules precipitated in the cell cytoplasm The activity was graded as + when the cell nuclei were visible through the mass of black granules in all cells The activity was designated as +++ when the nuclei were not visible (Fig 4) while ++ was designated when some of the nuclei were visible through the mass of black granules (Figs 2 and 5)

The degree of degeneration and regeneration of the SD active parietal cells was estimated by measuring the longitudinal extension of the injured area where the SD active parietal cells were absent (defect in SD active parietal cells Fig 1) The thickness of the mucosal layer containing parietal cells was measured with a graded



Fig 1

Drawing of a section of gastric mucosa showing the measurements made of the parietal cell layer L = length (total longitudinal extension) D = defect at the site of heating T = thickness (depth)

ocular diaphragm with 0 3 mm interval in the full length within the sections, excluding the ulcer defect (Fig 1) In each section also the longitudinal extension of the layer containing SD active parietal cells was determined, including the injured area (Fig 1)

RESULTS

In control mice the average thickness of the mucosal layer containing SD active parietal cells was 0 2 mm (standard deviation ($s = \sqrt{\frac{\Sigma(x^2)}{n}} =$ 0 06), and the average longitudinal extension of this layer was 3 4 mm (s 1 4) (Table 2) In these animals the average value of the pH in the gastric contents near to the mucosal surface was 2 2 (s 0 34), and the degree of succinic dehydrogenase activity in the parietal cells was ++ in 11 and + in 2 mice (Table 1)

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5 "	0 90	0 32	0 08	0 02	3 0	0 6	10
7 "	1 10	0 50	0 08	0 06	2 9	1 6	6
2 weeks	0 93	0 48	0 11	0 06	2 5	1 2	19
4 "	0 60	0 36	0 13	0 08	3 0	1 2	15
13 "	0 84	0 36	0 07	0 06	2 9	1 4	8
25 "	0 70	0 14	0 09	0 04	2 7	0 8	7



Fig. 2

Gastric mucosa of a control mouse. Normal SD activity (designated as ++) in the parietal cells. Cryostat section. MTT technique. × 200

In the operated animals pronounced changes were seen. A swelling of the gastric mucosa appeared in the heated area one half to one hour after the operation. The vessels were heavily dilated and engorged with blood, and in some of the animals a central excavation of the mucosa was seen. This excavation became more pronounced 3 to 24 hours after the operation, when also minimal haemorrhages were found. Three days after the operation the excavation was sharply outlined with a whitish grey granulation tissue appearing at the bottom of the ulcer area. Five days after the operation the granulation tissue had filled most of the ulcer, and after seven days the ulcer area was visible to the naked eye as a greyish spot the size of a pinhead with irregular and uneven borders against the normal mucosa. In most animals the site of the previous ulcer was still found 25 weeks after the operation.



Fig 3

The heated area of the gastric mucosa of a mouse killed twenty four hours after the thermal lesion Progressive loss of SD activity from the superficial to the basal part where still a narrow layer of SD active parietal cells are seen disrupted by oedema Cryostat section MTT technique × 200

Microscopical examination confirmed that normal parietal cells have a much higher degree of SD activity than the other cells of the gastric mucosa (Fig 2) A decrease of SD activity was generally associated with morphological signs of degeneration of the parietal cells while complete absence of activity indicated necrosis of these cells

In the heated area a pronounced edema was found in the first 24 hours after the operation and a progressive decrease in SD activity of the parietal cells was observed during this time generally extending from the superficial and central part of the corresponding mucosa towards the base and to the sides (Fig 3) The demarcation was not distinct until one to three days after the heating when the mucosal edges contrasted sharply against the necrotic cellular debris After the sloughing the ulcer area became covered by new epithelium Later new



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Fig 5

Cryostat sect on from the gastric mucosa of a mouse 4 weeks after the thermal lesion In the area of the former ulcer (left) no SD active parietal cells One of the glandular tubules is heavily dilated SD activity of parietal cells designated as ++ MTT technique × 200

cells was 1 1 mm (s 0 50) and 25 weeks after 0 7 mm (s 0 14) This difference was however not statistically significant (P 0 10 0 05)

The total longitudinal extension of the layer containing parietal cells was not significantly different in the control and and experimental series of mice But the average thickness of this layer was decreased in the series of mice with ulcer when compared to control mice (Table 2) The pH of the gastric content was increased most significantly when the degree of succinic dehydrogenase activity was decreased i e during the first hour after operation (Table 1, P 0 001) Thereafter the pH decreased difference from normal values

COMMENTS AND CONCLUSIONS

The results seem to show that an early effect of heating is a local disappearance of SD activity followed by degeneration and necrosis of



Fig 4

Margin of the heated area twenty four hours after the thermal lesion SD activity designated as +++ *in the surrounding* parietal cells This layer is interrupted by an area almost totally *devoid of* SD active parietal cells and with dilated glands The cells in these glands show a SD activity similar to that of the cells in the superficial layer (*ryostat section* MTT technique × 200

glandular structures were formed, some of which were dilated more or less irregularly This newly formed epithelium showed a similar low SD-activity as that found in normal chief and mucous *cells* (*Fig 5*) No parietal cells were observed in the area of the former ulcer

The average size of the area in which SD active parietal cells were absent, increased until the 7th day after operation whereafter no significant reduction was found during the following 24 weeks (Table 2 and Fig 6) Thus one hour after the operation the average size of the defect of SD-active parietal cells was 0 15 mm (s 0 14) *Twenty-four* hours after the operation the defect of SD-active parietal cells was 0 32 mm (s 0 30) and 3 days after the width of the defect was 0 9 mm (s 0 26) Seven days after the operation the defect of SD-active parietal

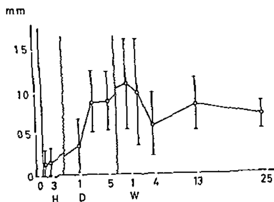


Fig 7

Size (length) of local defect in parietal cell layer at various stages following heating (cfr Table 2) The vertical lines represent one standard deviation (s) The observation time is designated by H (hours) D (days) and W (weeks)

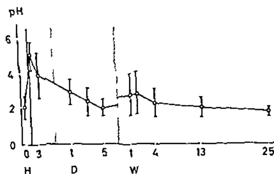


Fig 8

The pH of gastric content near the mucosal surface The drawn line represent average values and the vertical lines represent one standard deviation (s)

which seems to be similar to that of the superficial layer of the gastric mucosa grows quickly over the ulcer area But no SD active parietal cells are found within this region Thus after 25 weeks the size of the mucosal area in which SD active parietal cells were absent was within the same range as that found 3 to 7 days after the operation This finding shows that no differentiation to parietal cells occurs within the period of investigation neither from epithelial cells migrating from the surrounding mucosa nor from the neighbouring parietal cells outside the ulcer A slightly decreased size of the defect of the area containing SD active parietal cells was observed after 25 weeks pointing to a slight contraction of the scar The difference was however not statistically significant

The early effect of heating seems also to be followed by a general decrease of the parietal cell mass Thus the depth (thickness) of the

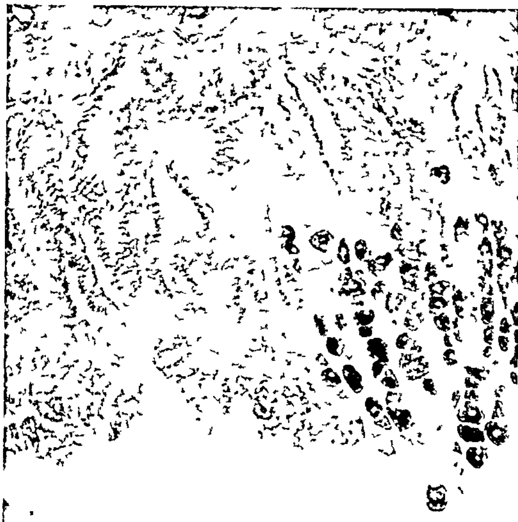


Fig C

Cryo tat secti n from the gastric mucosa of a mouse 6 m nths after tl e thermal les on In tl e area of the f rmer ulcer (left) no SD active ı arietal cell Ep thel al cells arrai ge l ın glan l lar tı l ıles w tl SD act v ty milar to that of cl ef or sı ı erf ıal ep thelial cells (r gl t) MTT techn q c × 200

parietal cells This is first seen in the superficial part of the gastric mucosa This finding may indicate that the ulcerogenic effect is due to vascular disturbances rather than to local heat

The results further show that thermal action confined to a small area of the gastric wall causes an increase of pH of gastric content near the mucosal surface the highest values being found one to three hours after the operation At this time the SD activity was decreased not only within but also outside the ulcer area This finding may indicate that the local thermal lesion may cause a more general disturbance leading to decrease in SD activity and in gastric production of acid

This early effect of heating is followed by sloughing and subsequent granulation and epithelial regeneration locally A new epithelium

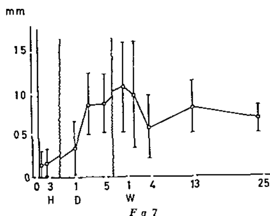


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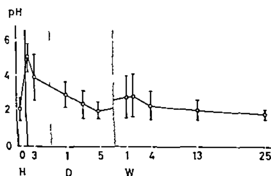


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The early effect of heating seems also to be followed by a general decrease of the parietal cell mass Thus the depth (thickness) of the

parietal cell layer outside the ulcer area was considerably reduced already on the 3rd–5th day after operation and did not regain its original size during the observation period of 25 weeks A normal SD activity within parietal cells was, however, regained and the pH of the gastric content decreased to values not significantly different from those of control animals This finding suggests the possibility that local heating may cause a more general damage of the gastric mucosa, the consequence of which may be a reduction of the total parietal cell mass

SUMMARY

The parietal cells of the gastric mucosa have been studied in mice following the production of a gastric ulceration by gentle local heating of the peritoneal surface of the fundus (55° C for 15 seconds)

One hour after the thermal lesion the succinic dehydrogenase (SD) activity was decreased not only in parietal cells of the ulcer area, but also in the other parietal cells of the stomach, as evidenced by examination of full-length sections along the anterior wall of the stomach The decreased SD-activity was accompanied by an elevated pH of the gastric contents

In the heated area, the earliest sign of retrogressive changes was a loss of SD-activity of the parietal cells This was first apparent in the superficial part, *i e* that most remote from the heat source Following necrosis and sloughing of the epithelium there occurred regeneration and formation of primitive glands, lacking parietal cells The size of the area devoid of parietal cells was not significantly altered during the course of the observation period of 6 months, thus indicating that the parietal cells are not regenerated neither from the epithelial cells in the ulcer area, nor from the parietal cells of the surrounding mucosa

Outside the heated area a reduced depth (thickness) of the parietal cell layer was found from the 3rd day after the operation, persisting through the observation period This observation points to the possibility that a local lesion may cause extensive disturbances leading to a permanent decrease of the parietal cell mass

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FROM THE DEPARTMENT OF MEDICINE, UNIVERSITY OF LUND ALLMÄNNA SJUKHUSET, MALMÖ, SWEDEN (HEAD PROFESSOR JAN WALDENSTRÖM M.D.)

THE EFFECT OF INDUCED FIBRINOLYSIS AND VARIOUS SUBSTANCES ON THE MAST CELLS IN THE PERITONEAL FLUID AND MESENTERY OF THE RAT

By

STIG CRONBERG and INGA MARIE NILSSON

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Anaphylaxis and various toxic and allergic conditions are accompanied by release of histamine and other amines, induction of fibrinolysis and an increased activity of various enzymes in the blood (34, 35 and others). Mast cells contain histamine (32), heparin (19), in some species 5-hydroxytryptamine (5, 30) and have also been reported to contain proteolytic enzymes (4, 15) and fibrinolytic activating substances (12, 13). The mast cells are known to release their granules after treatment with histamine liberators (14, 17, 26, 31), with peptone (21, 22, 27, 40) and in various anaphylactic disorders (6, 7, 8, 21, 25). According to some authors dextran degranulates mast cells in the rat (9, 10, 22, 36) but *Lagunoff & Benditt* (24) found no such effect on isolated mast cells. Histamine and 5-hydroxytryptamine have also been studied for their effects on the mast cells with conflicting results (1, 3, 22, 31, 36, 38, 39).

Especially *Ungar* (37) and his school have stressed that anaphylactic shock might be produced by induction of fibrinolysis and activation of various proteolytic enzymes with a secondary liberation of histamine.

The proteolytic enzyme trypsin has been reported to release histamine (2, 11, 33) and according to *Rocha e Silva* (33) it might play an essential rôle in the causation of anaphylactic shock. *Keller* (22) noted morphological changes in the mast cells after trypsin, but *Högberg & Uvnäs* (20) found no disruption.

The aim of the present investigation was to study the effect on the mast cells of fibrinolysis inducing agents, the amines histamine and 5-hydroxytryptamine, and some other substances of interest.

MATERIAL AND METHODS

Male and female albino rats weighing 150–350 g were used. The substances to be tested were given intraperitoneally or intravenously. The substances used were:

5-hydroxytryptamine. Serotonin from 'Roche', Basle, Switzerland was given in doses of 1 and 10 mg dissolved in 0.5 ml isotonic sodium chloride.

This study has been supported by a grant from *Hesselman's Foundation*.

Histam ne A commercial preparation from "ACO Stockholm Sweden 0 5 mg of histamine hydrochloride dissolved in 0 5 ml in isotonic sodium chloride

A lrenal ne A commercial preparation from ACO Stockholm 0 5 mg in 0 5 ml isotonic sodium chloride w th preservatives added

Nora lrenaline A commerc al preparation Norexadrin conc " from Astra Soder talje Sw den The preparation contained preservatives 0 1 0 5 mg were administered

Streptokinase (SK) Kabikinas supplied by Kabi" Stockholm 50 000 units d ssolved in 1 1 5 ml isotonic sodium chloride

Plasminogen A preparation of human plasminogen and proactivator obtained

brook England 100 mg d ssolved in isotonic sodium chloride

Dextran was obtained from Pharmacia" Uppsala Sweden Two fractions were used a 10 per cent solution of dextran of high molecular weight with a dens ty

a u u a dose of 1 ml

Peptone Proteose peptone No 3 from Difco" was used in doses of 25 and 200 mg dissolved in 0 5–1 ml isoton c sodium chloride

Heparin A commercial preparation fr m Vitrum Stockholm 1 ml containing 50 mg = 5000 IU was given

In some experiments a combination of two substances was used When possible the dos s of the solution injected was kept at 0 5–1 ml

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EXPERIMENTAL

Effect of Amines

Histamine and *5 hydroxytryptamine* intraperitoneally caused a marked increase in the amount of the peritoneal fluid and a dilatation of the vessels but did not disrupt the mast cells which occurred in normal number and appearance (Tables 1–3)

Adrenaline or *noradrenaline* given intraperitoneally caused likewise no disruption or degranulation of the mast cells

Effect of Fibrinolysis Promoting Substances

Streptokinase alone given intraperitoneally and in a large dose did not have any noxious effect on the mast cells in the peritoneal fluid or in the mesentery (Tables 1–3) No fibrinolytic activity developed

TABLE 1

Percentage Distribution of Different Types of Mast Cells According to Degree of Disruption 1½ Hours after Injection of the Test Solution.

Substance	Dose	Route	Types of mast cells *			
			A	B	C	D
O	–	–	94	6	0	0
Sodium chloride	0 9 % 0 5 ml	i p.	85	13	1	1
5-OH-tryptamine	10 mg	"	90	8	2	0
5-OH-tryptamine	1 mg	"	89	11	0	0
Histamine	0 5 mg	"	96	4	0	0
Adrenaline	0 5 mg	"	84	16	0	0
Noradrenaline	0 1–0 5 mg	"	89	11	0	0
Streptokinase (SK)	50,000 units	"	70	25	5	0
Plasminogen	400 mg	"	23	29	29	19
Plasminogen	50 mg	"	74	23	3	0
Plasminogen + SK	400 mg + 50,000 u	i.v.	87	13	0	0
Plasminogen + SK	400 mg + 50 000 u	i p	33	53	14	0
Plasminogen + SK	50 mg + 50 000 u	"	79	19	2	0
Plasminogen + ε-ACA	400 mg + 100 mg	"	9	28	40	23
Plasminogen + heparin	400 mg + 50 mg	"	42	27	13	18
Urokinase	4,500 units	"	81	17	2	0
Trypsin	5 mg	"	91	8	1	0
Nicotinic acid	25 mg	"	74	25	1	0
ε-ACA	100 mg	"	84	16	0	0
Dextran low mol wt	150 mg	"	82	17	1	0
Dextran high mol wt	100 mg	"	72	25	3	0
Albumin	200 mg	"	84	16	0	0
Heparin	50 mg	"	77	21	2	0
Peptone	200 mg	"	0	3	33	64
Peptone	25 mg	"	26	10	61	3
Peptone + ε-ACA	200 mg + 100 mg	"	4	5	38	53
Peptone + heparin	200 mg + 50 mg	"	39	39	17	5

* Explanation of symbols
A — Intact mast cells
B — Whole mast cells except for a few extracellular granules
C — Disrupted mast cells with the central part of the cell still remaining
D — Totally disrupted mast cells

after infusion of streptokinase alone *Streptokinase activated plasminogen* was prepared by adding 400 mg of human plasminogen to the dissolved streptokinase dose (50,000 units) This mixture was incubated at 37° C for 30 minutes After *intravenous* administration of the streptokinase activated plasminogen a marked fibrinolytic activity developed, and no blood clots formed within 1½ hours of the injection The mast cells in the mesentery and peritoneal fluid appeared normal When the same dose of streptokinase activated plasminogen was given *intraperitoneally*, more cells with released free granules and disrupted mast cells could be seen than in the controls (Table 1) even though the mast cells were not disrupted to such an extent as after administration of hista-

TABLE 2

Differential Counts of the Cells in the Peritoneal Fluid 1½ Hours after Injection of Test Solution

Substance	Dose	Route	Mast cells	Mononuclear cells with phagocyt granules	Mononuclear cells	Leucocytes	
						eos	neutr
0			2	8	71	19	0
Sodium chloride	0 9 % 0 5 ml	i p	3	3	85	9	0
5 hydroxytryptamine	10 mg		5	3	59	32	0
5-hydroxytryptamine	1 mg		1	0	74	25	0
Histamine	0 5 mg		3	0	90	7	0
Adrenaline	0 5 mg		2	0	94	4	0
Noradrenaline	0 1 0 5 mg	„	4	0	84	13	0
Streptokinase (SK)	50 000 units		2	0	78	20	0
Plasminogen	400 mg		0	1	98	0	1
Plasminogen	50 mg		2	16	64	17	1
Plasminogen + SK	400 mg + 50 000 u	i v	1	0	98	1	0
Plasminogen + SK	400 mg + 50 000 u	i p	1	0	99	0	0
Plasminogen + SK	50 mg + 50 000 u		6	25	28	35	6
Plasminogen + ε ACA	400 mg + 100mg		5	50	42	2	1
Plasminogen + heparin	400 mg + 50 mg	„	1	35	61	3	0
Urokinase	4 500 units		3	1	32	18	45
Trypsin	5 mg	„	4	1	67	25	3
Nicotinic acid	25 mg		3	0	82	15	0
ε ACA	100 mg	„	1	1	95	2	0
Dextran low mol wt	150 mg		0	0	99	1	0
Dextran high mol wt	100 mg		0	2	97	1	0
Albumin	200 mg		1	0	81	18	0
Heparin	50 mg	„	4	0	89	7	0
Peptone	200 mg		0	4	55	26	14
Peptone	25 mg		2	28	11	31	28
Peptone + ε ACA	200 mg + 100 mg		1	2	68	1	28
Peptone + heparin	200 mg + 50 mg		1	2	87	9	1

mine liberators After one day most of the mast cells appeared normal but regenerating cells and phagocytized mast cell granules were also observed This intraperitoneal injection did not produce any effect on the blood fibrinolytic system

In another series of experiments the streptokinase activated plasminogen was prepared by adding a smaller dose of human plasminogen, 50 mg to the same dose of streptokinase as in the above experiments After intraperitoneal injection of this smaller dose the mast cells in the mesentery and peritoneal fluid appeared normal, though phagocytosis of mast cell granules was observed in the peritoneal fluid Intraperitoneal administration of *plasminogen* without streptokinase produced similar changes (Table 1) A dose of 400 mg produced a moderate but

TABLE 3

Differential Counts of the Cells in the Peritoneal Fluid 24 Hours after Injection of Test Solution

Substance	Dose	Route	Mast cells	Mononuclear cells with phagocyt granules	Mononuclear cells	Leucocytes	
						Eos	Neutr
5-hydroxytryptamine	10 mg	i p	2	0	85	13	0
5 hydroxytryptamine	1 mg	„	8	0	56	36	0
Histamine	0 5 mg	„	2	0	96	2	0
Adrenaline	0 1 mg	„	3	0	75	22	0
Noradrenaline	0 1 mg	„	3	0	75	21	0
Streptokinase (Sk)	50 000 units		1	0	94	4	1
Plasminogen	400 mg	„	1	1	84	6	8
Plasminogen	50 mg	„	0	0	96	3	1
Plasminogen + Sk	400 mg + 50 000 u	i v	3	0	64	32	0
Plasminogen + Sk	400 mg + 50,000 u	i p	0	3	95	1	1
Plasminogen + Sk	50 mg + 50 000 u		1	0	92	6	0
Urokinase	4 500 units		0	0	61	4	35
Trypsin	5 mg	„	3	0	50	40	8
Nicotinic acid	25 mg		4	0	72	24	0
ε-ACA	*100 mg*		*1*	*0*	*91*	5	2
Dextran low mol wt	150 mg	„	0	3	85	1	10
Dextran high mol wt	100 mg		0	1	98	1	0
Albumin	200 mg		4	11	48	30	8
Heparin	50 mg		1	1	84	13	1
Peptone	200 mg	„	0	4	55	26	14
Peptone	25 mg		1	0	74	9	16
Peptone + ε ACA	200 mg + 100 mg		0	3	51	3	43

definite increase of disrupted cells with released granules When the dose was lowered to 50 mg, however, the mast cells appeared normal, even though some phagocytes with mast cell granules were seen Simultaneous injection of ε-aminocaproic acid in a dose of 200 mg did not prevent morphological changes produced by 400 mg plasminogen When heparin was injected together with 400 mg plasminogen, disrupted mast cells with released granules were also found

Urokinase given intraperitoneally had no demonstrable effect on the mast cells Nor did it interfere with clot formation An invasion of neutrophil leucocytes in the mesentery and peritoneal fluid was, however, observed

Trypsin given intraperitoneally produced no effect on the mast cells

Nicotinic acid given intraperitoneally had no harmful effect on the mast cells

ε-aminocaproic acid a strong inhibitor of fibrinolysis, was given intraperitoneally without any effect on the mast cells

Effect of Albumin, Dextran, Heparin and Peptone

Albumin, dextran of high or low molecular weight or *heparin* given intraperitoneally produced no subsequent changes in the mast cells (Table 1).

Peptone given intraperitoneally, on the other hand, produced extensive disruption of the mast cells, especially after injection of 200 mg. After 1½ hours the granules were scattered around the cells and the smears of the peritoneal fluid showed free granules and phagocytosis. After one day regenerating mast cells were seen in the mesentery. The disruption was less extensive when heparin was added to the peptone before injection (Table 1). ε-aminocaproic acid did not, however, inhibit the changes.

DISCUSSION

5-hydroxytryptamine and histamine produced no morphological changes in the mast cells. The degranulation reported by some authors might have been due to the noxious effect of vital staining with toluidine blue (18). Adrenaline and noradrenaline likewise had no harmful effect after intraperitoneal injection. In another experiment in hamsters (16) local injection of adrenaline was found to produce local necrosis with secondary destruction of the mast cells as well as of other cellular elements.

The effect of fibrinolysis on the mast cells was studied. After intravenous injection of 50 000 units of streptokinase no marked fibrinolysis developed, however. This dose of streptokinase given intravenously or intraperitoneally produced no effect on the mast cells. After intravenous administration of streptokinase activated human plasminogen fibrinolysis developed, but only after injection of such a large dose as 400 mg of plasminogen to 50 000 units of streptokinase. This dose had *no effect* on the mast cells. Intraperitoneal administration of the same dose of streptokinase activated plasminogen or plasminogen alone caused a moderate increase of extracellular granules and disrupted mast cells, but not fibrinolysis in the circulatory blood. The mast cell reaction was *not* abolished by ε-aminocaproic acid and we believe that the reaction must be considered unspecific and not secondary to fibrinolysis. Urokinase, which will also produce fibrinolysis although by another mechanism, *did not* destroy the mast cells. Nor did trypsin produce any changes.

Albumin, dextran or nicotinic acid were not found to produce any mast cell changes in these experiments.

As known, peptone disrupted the mast cells. ε-aminocaproic acid which is known to inhibit fibrinolysis did not prevent the disruption of the mast cells, an observation lending further support to the view that the fibrinolysis often observed in peptone shock (34) is not primary to the disruption of the mast cells. This is also in agreement with the

results of *Nilsson et al* (28) who found that ε-aminocaproic acid did not prevent the elicitation of anaphylactic shock in a hypersensitive patient Heparin did not disrupt the mast cells and had a moderate protective effect against the mast cell disruption produced by peptone, but it did not inhibit the more moderate changes produced by large doses of plasminogen

SUMMARY

In this study the effect on the mast cells of various fibrinolysis inducing agents and of the amines histamine, 5-hydroxytryptamine adrenaline and noradrenaline and some other substances of interest have been studied The amines had no effect on the mast cells Large doses of streptokinase had no effect on the mast cells, but large doses of plasminogen injected intraperitoneally alone or together with streptokinase produced a moderate increase in released mast cell granules Otherwise the fibrinolytic agents did not produce mast cell changes Albumin, dextran and heparin were also ineffective but peptone produced an intense disruption of mast cells which could not be prevented by ε-aminocaproic acid, an inhibitor of fibrinolytic activity

The results do not indicate that the amines tested or the induction of fibrinolysis will cause disruption of the mast cells

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FROM THE CENTRAL HOSPITAL, DEPARTMENT OF PATHOLOGY, RANDERS DENMARK HEAD (TEMPORARY) J CHR GIERTSEN, M D

PULMONARY CARCINOMATOUS THROMBO-EMBOLISM

By

J CHR GIERTSEN and E SOMMER HANSEN

Received 19 viii 63

In 1903 *Schmidt* (15) showed that the lungs—although macroscopically normal—in a considerable number of abdominal cancer cases (15 out of 41) revealed extensive tumour embolism on microscopical examination. His findings suggested that these tumour emboli were either destroyed by the organization of the subsequently formed thrombus, or that they might grow through the capillaries and reach the veins, whereby the path was laid open for a further spread of the tumour via the systemic circulation. Shortly afterwards *Teacher* (18) reported the healing by thrombus formation of pulmonary emboli from a chorion-epithelioma.

Since then several reports have corroborated *Schmidt*'s interpretation of his findings, both through postmortem (7, 8, 10, 14, 16) and experimental (3, 7, 9, 17, 19) observations.

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Saphir (14) stressed that the tumour emboli are not actually organized, but they disappear and are replaced by the organizing thrombus. He further emphasized that very often the results of this organization cannot be distinguished from pulmonary arteriosclerosis. He therefore suggested that tumour emboli should be suspected if microscopical examination disclosed an unexplained pulmonary arteriosclerosis in a cancer patient.

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CASE HISTORY

56 year-old male metal grinder Ten years previously he was treated in the

... years he felt well apart from a tendency to hypoglycaemic seizures

One year before death his right eye was injured by a metal splinter and a pterygium developed Since then he suffered from recurrent rhinitis

At a chest control 5 months before death he complained of fatigue but displayed no pulmonary distress

Two months before death he ...
not inhale enough air into his l...
At the same time he noticed ter...
tenderness of the larynx The f...
Four weeks b...

Physical fi...
plexion ...
... abnormal
palpable alon...
hard but not ...
b th axillae ...
enlargement

... of the chest 18 days before death ... that Roentgenological examination

The hilar regions ...
clear Both diaphragm ...
laterally on both s...
1956) Normal heart cont ur ... on the left side since

Biopsy of a lymph node from the neck ...
tissue had been replaced by a no...
by strands of fibrous tissue The...
pattern The cells were definitely...
polyhedral, rather small with a...
varying amount of eosinophilic ...
presenting varying stages of cell...
seen

A simultaneous biopsy from the ...
perivascular lymph ...

The findings gave r...

FROM THE CENTRAL HOSPITAL DEPARTMENT OF PATHOLOGY, RANDERS DENMARK HEAD (TEMPORARY) J CHR GIERTSEN, M D

PULMONARY CARCINOMATOUS THROMBO-EMBOLISM

By

J CHR GIERTSEN and E SOMMER HANSEN

Received 19 viii 63

In 1903 *Schmidt* (15) showed that the lungs—although macroscopically normal—in a considerable number of abdominal cancer cases (15 out of 41) revealed extensive tumour embolism on microscopical examination His findings suggested that these tumour emboli were either destroyed by the organization of the subsequently formed thrombus, or that they might grow through the capillaries and reach the veins, whereby the path was laid open for a further spread of the tumour via the systemic circulation Shortly afterwards *Teacher* (18) reported the healing by thrombus formation of pulmonary emboli from a chorion-epithelioma

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At a chest control 5 months before death he complained of fatigue, but displayed no pulmonary distress

[illegible] Slight tenderness of the larynx on palpation, but no enlargement of the thyroid gland was noted

[illegible]

perivascular

The findir [illegible]

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Fig 1

The thyroid gland d ffusely inf ltrated by an und fferent ated carcinoma probably the pr mary tumour H + F × 80



Figs 2 3

Fig 2 An arteri le occluded b a fresh tum ur embolus Note m tosis in centre H + F × 210

Fig 3 An art riole w th a small tumour embolus surr unded by a thrombus in an in tial state of rganizati n H + E, × 80

of an—admittedly doubtful—positive Alcian Blue reaction a mucinous adenocarcinoma was hinted

Clinical course The patient was transferred to the Department of Irradiation Therapy *There was still no dyspnoea at rest nor any cyanosis A slight enlargement* of the thyroid gland was noted A palliative irradiation of the enlarged lymph nodes was started A few days later the patient became acutely ill He was cold and pale and complained of pain below the right curvature The pulse rate increased and the blood pressure decreased Death ensued about four hours later

Autopsy findings (relevant findings only) 56-year-old male in medium state of nutrition The thyroid gland was enlarged (weight 56 g) *and adherent to the larynx On palpation the gland felt hard,* and sectioning showed that it was diffusely infiltrated with slightly nodular tumour tissue There were numerous enlarged, hard lymph nodes along both sides of the trachea and in the lung hilus, but not below the diaphragm

The lungs were rather large and slightly oedematous, but they contained a normal amount of air There was no palpable tumour, but some minute nodules, smaller than a pin-head, could be felt The *bronchi* were normal The heart was normal with no sign of a cor pulmonale Apart from a slight hyperplasia of the prostate gland there were no other pathological findings It should be emphasized that no gastrointestinal tumour was found, and, apart from the lymph node involvement, no metastases were found

Microscopical examination The paratracheal and hilar lymph nodes revealed essentially the same changes as the biopsy lymph node

In the thyroid gland (Fig 1) a diffuse tumour growth was seen, partly infiltrating the stroma, and partly growing in lymphatics The tumour was undifferentiated and showed no glandular pattern, but in some areas a suggested papillary growth was seen Degeneration and necrosis were present in many areas The cells displayed a pronounced atypia and pleomorphism, and were identical with those found in the lymph node biopsy No definate primary nodule was found

The bronchi were normal, and a slight emphysema was present in the lungs In addition, the small arteries and arterioles with lumen diameters up to about 400 μ revealed extensive changes Practically no vessel remained unaffected In some arterioles recent tumour emboli were found (Fig 2) In others the emboli were enclosed in thrombi in varying stages of organization (Fig 3), with distinct signs of destruction of the tumour cells, as the cytoplasm was irregularly *outlined*, and the nuclei shrunken or disintegrated (Fig 4) Numerous arterioles were completely occluded by the organized thrombus (Fig 5), whereas recanalization had occurred in some (Fig 6) Fresh tumour emboli could be seen in some of these recanalized vessels (Fig 7) No cellular reaction was seen, except for a negligible lymphocytic infiltration in some of the arterial walls A rather large artery was partly occluded by an embolus with a surrounding *thrombus apparently adhering quite accidentally* to the arterial wall (Fig 8), but serial sections revealed that it was actual-



Figs 8 9

Fig 8 An arteriole with a tumour embolus enclosed in a thrombus adhering apparently accidentally to the arterial wall H + E, × 80

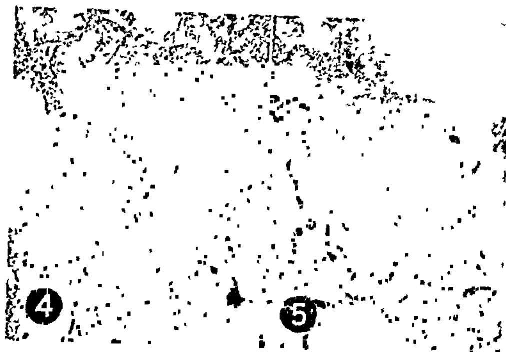
Fig 9 Serial sections show the thrombus situated at the ostium of a small branch of the arteriole, filled with tumour cells H + E × 80

ly situated at the ostium of a small branch filled with tumour cells (Fig 9) The other end of this large artery divided into several smaller branches, all of which were occluded Tumour cells were not seen to invade the vessel wall, nor were tumour cells observed in the capillaries or veins The perivascular lymph spaces were filled with tumour cells, so that a lymphangitis carcinomatosa was present, but these distended lymphatic vessels could obviously not have exerted any pressure on the arteries

DISCUSSION

Although no definate primary tumour was found, the findings were very suggestive of a primary thyroid carcinoma, in an undifferentiated papillary form, with extensive infiltration of the entire gland and invasion of the lymphatic vessels In most previous reports the primary tumour was an abdominal—mostly gastric—cancer (4, 8, 10, 15, 16) We are positive, however, that a gastric carcinoma was not present (as mentioned, the positive Alcian Blue reaction was doubtful)

It is not possible to ascertain how the tumour emboli reached the lesser circulation in our case They must of course have reached the upper vena cava in some way, either through direct invasion of veins, or via the thoracic duct The extensive lymphatic growth makes the second possibility most likely, and it is believed that after the tumour reached



Figs 4–5

Fig 4 Advanced organization of a thrombus The tumour cells are shrunken with pycnotic nuclei *H + F* × *80*

Fig 5 An arteriole with complete organization of the thrombus The tumour cells have disappeared *H + F*, × *80*



Figs 6 7

Fig 6 A thrombosed and recanalized arteriole H + F × *80*

Fig 7 A thrombosed and recanalized arteriole with a small fresh tumour embolus H + F, × 80

of the pulmonary artery and the conus arteriosus perivascular infiltration and evidence of some passive congestion The clinical diagnosis of tumour embolism is of course difficult, but the above-mentioned clinical picture seems suggestive if it occurs in a cancer patient Heart catheterization should prove valuable in the evaluation of the condition

The patient did never experience any symptoms suggestive of a sudden vascular catastrophe, except terminally, when he complained of pain below the right curvature The process has, therefore, probably consisted in a more or less continuous liberation of a small number of emboli rather than intermittent large showers The unexpected death is explained as a result of a complete respiratory collapse Possibly, a final shower of emboli represented a contributing factor

SUMMARY

A 56 year old male developed dyspnoea on exertion 10 weeks before death manifesting itself in a subjective feeling of air hunger which could not be explained by the findings at the physical examination as the air passages were free Carcinoma metastases were found in the lymph nodes of the neck The condition did not change and the patient died rather unexpectedly At autopsy a thyroid carcinoma with extensive lymphatic invasion was found including a pulmonary lymphangitis carcinomatosa In addition an extensive tumour embolism was *found in the lungs* The emboli had incited the formation of thrombi, which were organized During this process the tumour emboli were *destroyed* and disappeared leaving a completely occluded artery Recanalization with renewed tumour embolism was seen

The problems of pulmonary tumour embolism are discussed in view of the case findings

The unexpected death was ascribed to a respiratory collapse possibly initiated by a final shower of tumour emboli

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the thoracic duct, showers of tumour emboli were liberated and entered the left subclavian vein However, the thoracic duct was unfortunately not specially dissected

The further sequence of events is suggested in the microscopical description of the vascular changes in the lungs, and need not be repeated It should only be stressed that the findings in no way support *Brill & Robertson*'s (4) suggestion of a pressure effect of the distended lymphatic vessels on the arterioles

The simultaneous appearance of lymphangitis carcinomatosa and arterial tumour emboli led *Morgan* (10) to contend that the former was secondary to the letter, and not due to a retrograde spread from the hilar lymph nodes Our findings can neither support nor invalidate this suggestion, but the abscence of vessel wall invasion and of extra vascular growth seem significant As is well known, the common explanation of pulmonary lymphangitis carcinomatosa is a retrograde spread (21)

Our findings suggest that the tumour emboli do not adhere casually to the endothelial lining of the arterial wall, as serial sections showed that the thrombus was situated at the ostium of a small branch It seems more reasonable to suppose that the emboli follow the blood stream and get lodged in the artery by plugging of the lumen In our case tumour emboli of up to more than 20 cells were seen, lodging in lumen with a diameter of up to 400 μ

The tumour emboli in our cases were undoubtedly viable, as evidenced by the mitoses seen, but these are not in themselves proof of an intra vascular growth As is known, the lung is one of the predilection sites for metastatic thyroid growth Nevertheless, the tumour emboli were virtually strangled by the organizing thrombus, and no metastasis had developed The different factors of possible importance in deciding whether a tumour embolus will produce a metastatic growth or not are thoroughly discussed by *Willis* (21) Suffice it to say that he compares the tumour emboli to a tumour tissue culture, the viability of which depends on the medium The destruction of tumour cells by the formation of a thrombus that is organized should probably be regarded as a teleological process, although the ultimate effect on the individual may prove disastrous

Our patient's main and only symptom was dyspnoea on exertion, manifesting itself in a subjective feeling of air hunger This feeling could not be explained by the findings at the physical examination, as the air passages were free (the rhinitis present can hardly have been of significance), and the vital capacity was normal One may wonder at the fact that cyanosis was absent and that a secondary polycythaemia had not developed Roentgenologically the lung fields were clear *Morgan*'s (10) patient revealed a mottling of the upper two thirds of the lungs (this patient had been suffering from a chronic bronchitis for many years) *Brill & Robertson*'s (4) patient revealed a dilatation

FROM THE CENTRAL HOSPITAL DEPARTMENT OF PATHOLOGY RANDERS DENMARK HEAD (TEMPORARY) J CHR GIERTSEN M.D

MALIGNANT MELANOMA WITH METASTASES IN A 6 YEAR OLD BOY

By

J Chr Giertsen

Received 19 viii 63

Recent studies of pigmented tumours with clinico pathological correlation of the findings have added extensively to our knowledge and changed the concept of these tumours especially of those occurring before puberty

Thus *Spitz* (8) separated the juvenile melanoma from the malignant melanoma group and emphasized its benign character As this tumour may be found in adults and as the word melanoma may be suggestive of malignancy *McWhorter & Woolner* (6) suggested the term benign juvenile melanoma , a term that implies the existence, however, of a malignant variant of the lesion *Helwig* (4) proposed the name spindle cell and/or epithelioid nevus , which despite its cumbrousness is preferred by *Lund & Kraus* (5)

Further the fundamentally benign character of the blue naevus even of the cellular type, which may show a sarcomatoid appearance has been stressed by *Montgomery & Kahler* (7), *Dorsey & Montgomery* (3), and *McWhorter & Woolner* (6)

An extensive literature review of the recent papers has been given by *Beerman Lane & Shaffer* (2)

When viewed in the light of this new knowledge the diagnosis in many previous reports on malignant pigmented tumours in children has been questioned (1 3 5 6 7), because the documentation presented has been considered inadequate juvenile melanomas have been labelled malignant melanomas cellular blue naevi have been classified as malignant malignant melanomas have been mistaken for blue naevi and metastases from malignant melanomas have been called malignant blue naevi

Malignant pigmented tumours do occur in childhood however, although very rarely *Allen & Spitz* (1) reported 5 malignant melanomas Only 3 of these belonged to their own series (934 cases), giving a percentage incidence of less than 0 3 of all malignant melanomas of the skin and mucous membranes *McWhorter &* [illegible]

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Dr Giertsen s present adress Department of Pathology The National Medical Center in Korea The Scandinavian Mission APO 301 San Fransisco Calif USA

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Dr Giertsen s present adress Department of Pathology The National Medical Center in Korea The Scandinavian Mission APO 301 San Fransisco Calif USA

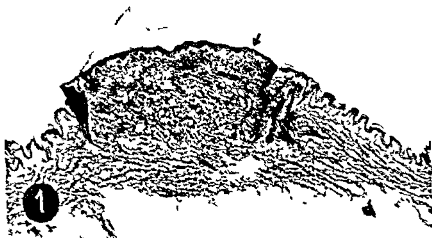


Fig 1

General view of the tumour slightly peripheral to the centre The free zone between the tumour and the subcutis is rather broad in this section The arrow points at the only intraepithelial cluster of naevus cells seen in the entire tumour H + E × 13



Fig 2

A central part of the tumour with large nests of ordinary looking naevus cells situated immediately beneath the epithelium Note increased number of clear cells H + E, × 210

they found 102 reports on malignant melanomas in childhood, but accepted the diagnosis in 13 cases only

Malignant blue naevi also seem to be very rare *Allen & Spitz* (1) reported 6 cases, 4 of which belonged to their series In addition, they reported 4 cases of cellular blue naevi with minute lymph node metastases *Dorsey & Montgomery* (3) reported 3 cases *Lund & Kraus* (5) reported 1 case of their own and mentioned some other cases, referring partly to the literature, and partly to personal communications

CASE HISTORY

A 6 year-old boy was admitted to the Department of Surgery, the County and Municipal Hospital of Grenaa, to have a mole removed, situated on the anterior aspect of the right upper thigh Since the mole had been noticed by the boy s parents 1 year previously, it had grown to about twice its original size

The tumour was excised, together with 2 regional lymph nodes in the right groin Smaller, indolent lymph nodes were noted in the other groin and in both axillae but none of these were excised A thorough search revealed no other tumours

Macroscopically the tumour measured 3 by 5 mm, and was raised about 1 mm above the skin surface The colour was dark blue, the surface shiny It was hairless, and sharply circumscribed from the surrounding skin The tumour apparently occupied the entire dermis, and showed a heavy blue-black pigmentation One of the lymph nodes appeared normal to the naked eye The other was blue black and consisted of 2 nodes bound tightly together by strands of fibrous tissue

Microscopically (Figs 1–7) the tumour was situated just beneath the epithelium, extending down through the dermis, leaving only a narrow zone adjacent to the subcutis free It was covered by a thin, flattened epithelium with small and inconspicuous rete pegs One single cluster of naevus cells was found lying within the epithelium These cells were uniform in size and revealed no particular atypia The entire tumour was serial-sectioned, and no more epithelial clusters of naevus cells were found The basal layer of the epithelium was slightly more pigmented than is normal In addition, a slightly increased number of clear cells was seen The epithelium was for the most part sharply separated from the underlying tumour, but in several areas a distinct continuity between the epithelium and the tumour tissue was observed In these areas the epithelial cells seemed to change imperceptibly into tumour cells and blend with these Just beneath the epithelium clusters of rather closely packed cells were found These clusters varied in size, but the cells were definitely like ordinary naevus cells The nuclei were round or oval, rather small The cytoplasm was slightly eosinophilic with indistinct borders and in some cells it was vacuolated No giant cells could be seen A definite atypia was present in some cells These were slightly larger, with large, irregularly polyhedral nuclei, occasionally showing hyperchromasia This superficial area with nests of atypical naevus cells and bridges from the epithelium to the nests, was li-



Fig 1

C neral — — f t ... one between ...points at the ... + E × 13



Fig 2

A central part of the tumour with large nests of ordinary looking naevus cells situated immediately beneath the epithelium Note increased number of clear cells H + E × 210

Fig. 3
A nest of slightly pleomorphic naevus cells H + E, × 520

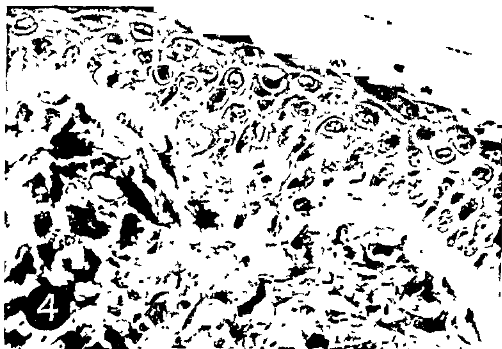


Fig. 4
An area with indistinct border between the epithelium and the tumour. The epithelial cells change imperceptibly into tumour cells H + E × 520

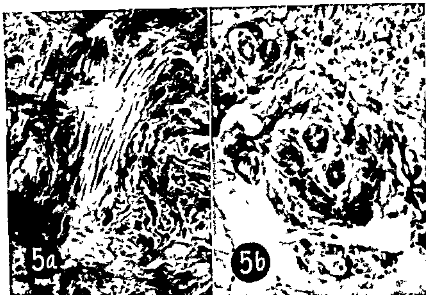


Fig 5
Growth of tumour cells (A) along a cutaneous nerve and (B) around sweat glands H + F, × 210

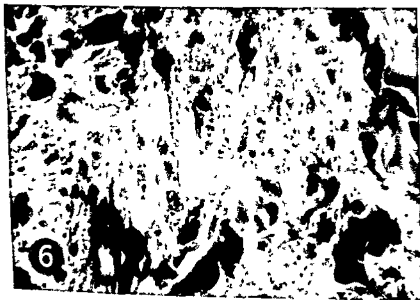


Fig 6
From the deeper part of the tumour Epithelioid cells and transitional forms to spindle cells surrounded by melanophages H + F × 520

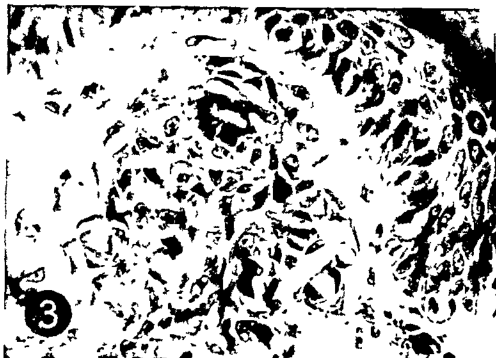


Fig 3
A nest of slightly pleomorphic naevus cells H + E × 520

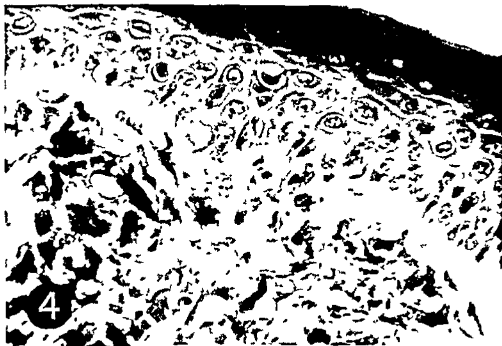


Fig 4
An area with indistinct border between the epithelium and the tumour The epithelial cells change imperceptibly into tumour cells H + E × 520

the above mentioned two cell types contained considerable amounts of melanin so as to completely obscure the nucleus These cells were undoubtedly melanophages Mitoses were only found occasionally and large areas had to be searched to find any There was no inflammatory reaction in the stroma

In the lymph nodes the lymphoid tissue was almost completely replaced by solid tumour tissue This was built up almost exclusively of epithelioid cells showing a very pronounced atypia and pleomorphism The cells were comparatively rich in cytoplasm which stained faintly eosinophilic They were round oval or polyhedral and varied markedly *in size* The largest nuclei were approximately 15 20 times the size of the smallest The nucleus was prominent with finely dispersed chromatin Many cells and nuclei showed bizarre forms and many giant cells and multinucleated cells were observed Occasional small bands of spindle cells were also seen The cells were rich in melanin Mitoses were found more frequently in the metastases than in the primary tumour but even here they had to be searched for

DISCUSSION

The tumour presented shows some of the growth characteristics of the blue naevus namely the growth around capillaries cutaneous nerves and sweat glands A blue naevus may be combined with a junctional naevus (5 6) and with an intradermal naevus (1) However the epithelioid cells and their transitional forms located deep in the tumour and the absence of dendritic processes suggest an epidermal rather than a dermal origin of the cells

The malignant character of the tumour is proved by the presence of metastases The tumour lacks 3 of the characteristics of the malignant melanoma however *viz* extensive intraepithelial growth abundancy of mitoses and inflammatory reaction The only intraepithelial cell nest found did not give any hint of malignancy *McWhorter & Woolner* (6) also found only one single intraepithelial nest in one of their malignant melanomas (Case No 5) but the malignancy of that tumour has not been proved by the occurrence of metastases A definite dermo epidermal activity is *indicated* in the present tumour however by the presence of an increased number of clear cells in the epidermis and the continuity between the epithelium and the tumour In this connection the importance should be stressed of ha ng —

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It is difficult however *to take* a firm standpoint as to the grade of malignancy *Lund & Kraus* (5) divide the malignant melanomas of

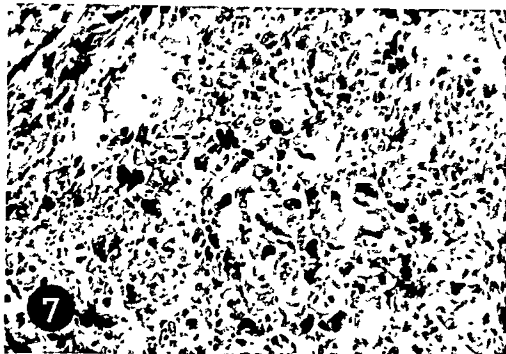


Fig 7

From the lymph node metastases Extreme pleomorphism with giant cells and multinucleated cells, a pattern not at all seen in the primary tumour H + F × 210

mited in extent, as shown by the serial-sectioning From this area the main bulk of the tumour grew, in the shape of a ball, deep down into the dermis In sections from the peripheral part of the tumour, a free zone could be found between the epithelium and the tumour

In the deeper part of the tumour a more variegated picture was observed Epithelioid cells were intermingled with spindle cells and transitional forms between the two The epithelioid cells were more atypical than those in the superficial cell nests, and showed a marked difference in size The largest cells contained a large, irregularly round or oval nucleus, often with a prominent nucleolus, but with rather sparse chromatin The smaller cells contained hyperchromatic nuclei The cell borders were indistinct or completely unrecognizable The epithelioid cells displayed no tendency to any particular pattern of arrangement The spindle cells had a long and slender, often rodlike nucleus with indistinctly outlined cytoplasm These cells were often arranged in small bundles, growing along coarse collagen bands Tumour cells of both kinds were also found to be arranged around capillaries, cutaneous nerves and sweat glands Reticulin staining revealed that some cells had small, inconspicuous fibrils, but definite dendritic processes could not be seen

The tumour was heavily pigmented The pigment showed the staining properties of melanin and was found both in the epithelioid cells and the spindle cells In addition some very large cells, intermingled with

the above-mentioned two cell types, contained considerable amounts of melanin, so as to completely obscure the nucleus These cells were undoubtedly melanophages Mitoses were only found occasionally, and large areas had to be searched to find any There was no inflammatory reaction in the stroma

In the lymph nodes the lymphoid tissue was almost completely replaced by solid tumour tissue This was built up almost exclusively of epithelioid cells, showing a very pronounced atypia and pleomorphism The cells were comparatively rich in cytoplasm, which stained faintly eosinophilic They were round, oval or polyhedral, and varied markedly in size The largest nuclei were approximately 15–20 times the size of the smallest The nucleus was prominent with finely dispersed chromatin Many cells and nuclei showed bizarre forms, and many giant cells and multinucleated cells were observed Occasional, small bands of spindle cells were also seen The cells were rich in melanin Mitoses were found more frequently in the metastases than in the primary tumour, but even here they had to be searched for

DISCUSSION

The tumour presented shows some of the growth characteristics of the blue naevus namely the growth around capillaries, cutaneous nerves and sweat glands A blue naevus may be combined with a junctional naevus (5, 6) and with an intradermal naevus (1) However, the epithelioid cells and their transitional forms located deep in the tumour, and the absence of dendritic processes, suggest an epidermal rather than a dermal origin of the cells

The malignant character of the tumour is proved by the presence of metastases The tumour lacks 3 of the characteristics of the malignant melanoma, however, *viz* extensive intraepithelial growth, abundancy of mitoses and inflammatory reaction The only intraepithelial cell nest found did not give any hint of malignancy *McWhorter & Woolner* (6) also found only one single intraepithelial nest in one of their malignant melanomas (Case No 5), but the malignancy of that tumour has not been proved by the occurrence of metastases A definite dermo-epidermal activity is indicated in the present tumour, however, by the presence of an increased number of clear cells in the epidermis, and the continuity between the epithelium and the tumour In this connection the importance should be stressed of having serial sections of even small tumours by which to obtain the correct impression of the anatomy of the tumour A further indication of activity and possible malignancy is the pleomorphism of the epithelioid cells located deep in the tumour, in spite of the small number of mitoses The correct designation for our tumour therefore seems to be a malignant melanoma with metastases

It is difficult, however, to take a firm standpoint as to the grade of malignancy *Lund & Kraus* (5) divide the malignant melanomas of

childhood into 3 categories, *viz* transplacental metastases from mother to child, malignant melanomas with fatal outcomes, and malignant melanomas with lymph node metastases but with indefinitely prolonged survival The fatal malignant melanomas reported by *Allen & Spitz* (1) and *McWhorter & Woolner* (6) showed an extreme degree of anaplasia On the other hand, *Lund & Kraus'* third category is characterized by discrete, microscopical lymph node metastases The present tumour lacked anaplasia, but showed large metastases visible to the naked eye, and these were extremely anaplastic Thus, the tumour does not fit exactly into either of the categories In view of the small number of mitoses it seems reasonable to place it in the third category, however The ultimate prognosis, of course, remains to be seen

One would, and probably should, hesitate to suggest malignancy in a tumour like ours from the histological appearance of the primary tumour alone, especially when it occurs in a child Nevertheless, the present tumour has metastasized On the other hand, even experts *cannot distinguish one third of the juvenile melanomas from the adult* malignant melanoma on the basis of the histological appearance alone (1), and these tumours are benign Thus, the histological features of the pigmented tumours in children obviously do not invariably reflect their biological potentialities, and we have still no absolute and conclusive histological evidence to rely upon when deciding whether such a tumour is malignant or not Therefore, in daily practice the pathologist will still have to be careful in the evaluation of some of the pigmented tumours in children, and guarded in his advice to the clinician

SUMMARY

A malignant melanoma with metastases in a 6 year-old boy is reported The tumour was situated on the anterior aspect of the right upper thigh, and metastases were found in the regional lymph nodes in the right groin The tumour was composed of atypical naevus cells *epithelioid and spindle forms, probably derived from the epithelium* The histological picture was semi-malignant only The metastases were almost exclusively composed of extremely pleomorphic epithelioid cells Because of the small number of mitoses the tumour was supposed to be of a low grade malignancy, with a fairly good prognosis It is stressed that the pathologist will have to be careful in the evaluation of the biological potentialities of some of the pigmented tumours occurring in children

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ADDENDUM

Recently Myhre E commented upon 4 malignant melanomas in children (Acta path et microbiol scand nav 59 184 188 1963)

Author s present adress Department of Pathology The National Medical Center in Korea The Scandinavian Mission APO 301 San Fransisco Calif USA

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FROM STATENS SERUMINSTITUT COPENHAGEN DENMARK
STREPTOCOCCAL DEPARTMENT CHIEF KNUD SKADHAUGE M D

INFECTION AND MALIGNANT TUMOURS

7 *Comparison between Extract (Intracellular Products) and Culture Filtrate (Extracellular Products) of Haemolytic Streptococci, in their Effect on Brown-Pearce Carcinoma in Young Rabbits*

By

EBBE AHRENSBURG CHRISTENSEN and EBBE KJEMS

Received 6 vii 63

Previous studies showed that the growth of Brown-Pearce carcinoma in young rabbits can be inhibited by treatment with haemolytic streptococci lysed by phage (4, 1). A similar effect was also obtained by treatment with cell-free extracts of the same strain of streptococci, prepared by grinding the bacteria with alumina (2).

In the present study, the tumour-inhibiting effect of extract from a Group A streptococcus (intracellular products) was compared with the effect of culture-filtrate (extracellular products) from the same streptococcal strain. Further, a determination was made of the number of infectious phage particles present in the streptococcal preparations used.

MATERIAL AND METHODS

Young male rabbits of a white Danish breed ("Copenhagen Whites" from 'Hvidesten', the breeding farm of Statens Seruminstitut) were used in the experiments. All the animals were between 8 and 11 days old at the time of transplantation. Transplantations were performed by means of intraperitoneal injection of a cell suspension of Brown Pearce carcinoma (4). Placebo treated control animals and rabbits that had received treatment with preparations of streptococci were from the same litter. The treatment consisted of intraperitoneal injections, the first one given two days after the transplantation. The period of treatment lasted three weeks with two or three injections per week.

The general health condition of the animals was judged on the basis of their increase in weight and on their appearance. All animals were weighed on the day of transplantation, three days later, subsequently once a week and finally on the day of death. The rapid growth of the tumours in the peritoneum and the appearance of metastases as early as the 11th day after transplantation made it possible to terminate the experiments between the 24th and 25th day after transplantation.

All rabbits were examined at autopsy by the same investigator using a standard technique. On the basis of the autopsy findings the animals were classified in three groups according to the occurrence of metastases (Fig 1 A) and in three groups according to the quantity of tumour tissue (Fig 1 B) (4).

The streptococcal preparations were made from the strain used in previous experiments, viz. *Streptococcus pyogenes* Group A Type 12 no 3465 (local isolation number). This strain is phage producing (8).

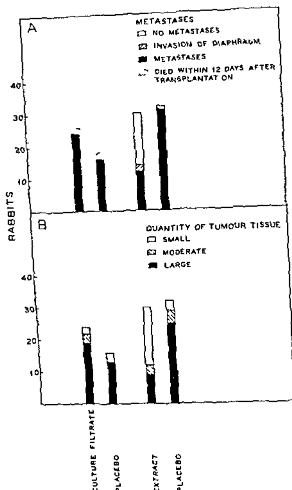


Fig 1 A and B
Metastases and quantity of tumour tissue at autopsy

The filtrate (extracellular products) was prepared by growing the streptococci in trypsin broth (12) for 24 hours The streptococci were removed by centrifugation followed by filtration through a Berkefeld filter Trypsin broth was used as placebo
The extract (intracellular products) was prepared by grinding the streptococci with alumina and extracting the mass with isotonic phosphate buffer saline pH 7 38 (2) Isotonic phosphate buffer saline was used as placebo
The infectious phage particles were counted by means of the usual technique (7)

RESULTS

A total of 26 animals received filtrate in doses as large as possible without affecting their general health condition, *i e* from 50 to 100 ml per animal Corresponding doses of trypsin broth were given to a total of 18 animals Two animals under treatment with culture-filtrate and

two animals receiving placebo died before the 12th day after transplantation, and could therefore not be compared with the other animals Treatment with filtrate had no effect on the growth of the tumour (Fig 1 A and B)

A total of 32 animals were treated with extract from haemolytic streptococci, and 32 animals received corresponding doses of buffer saline Two animals under treatment with extract died before the 12th day after transplantation, and could therefore not be compared with the other animals The dosage varied from 5 ml to 15 ml extract per animal, corresponding to between 0 5 and 1 5 g bacteria (wet weight) This treatment resulted in pronounced inhibition of the tumour in more than half the animals (Fig 1 A and B)

As mentioned, strain 3465 is a phage-producing strain, and one ml of the filtrates contained 6–40 × 10⁴ infectious phage particles However, no infectious phage particles could be demonstrated in the extracts

DISCUSSION

In the present experiments, no tumour-inhibiting effect could be demonstrated by filtrates (extracellular products) from a strain of haemolytic streptococci On the basis of the effect of the dosage employed it is possible to make an estimate of the maximum content of tumour-inhibiting factor that could have been present in the non-active filtrates in relation to the corresponding content of tumour-inhibiting factor in the extracts and in the lysates of streptococci used in previous studies (4, 1) Comparing the results of the present experiments with the results obtained in a large material of lysate-treated animals, where the dosage was varied (1), it can be estimated that the content of tumour-inhibiting factor in the filtrates was at most 25 per cent of that present in the lysates A comparison of this kind seems permissible since the experiments were carried out with the same tumour, the same breed of rabbits, the same experimental technique, and the same streptococcal strain was used for the production of lysates, filtrates and extracts

In the present experiments, an amount of 5–15 ml of extract showed tumour-inhibiting effect, while 50–100 ml of filtrate had no effect on the tumour The content of tumour-inhibiting factor in the culture filtrate has therefore been at most 10 per cent of that in the extracts On the other hand, it was not possible to compare the extracts and the filtrates on the basis of the amount of culture employed A volume of 5–15 ml of extract was prepared from the bacteria in 200–500 ml of culture, and thus corresponded to this volume of filtrate Such a volume, however, was 2 5 times the dose of filtrate that could be used in the treatment, if the general health condition of the animals was not to be affected

It was previously shown (4) that the tumour-inhibiting effect of a lysed streptococcal culture was the same whether the lysis was caused

by sewage phage (A 12, Maxted 1955), "strep" phage (7) or both phages The present experiments showed likewise that an inhibiting effect on Brown Pearce carcinoma of streptococcal preparations did not depend on the number of infectious phage particles in the preparations, as infectious phage particles could not be demonstrated in the tumour-inhibiting extracts

It seems a reasonable assumption, therefore, that the significance of "strep" and "sewage" phage for the tumour-inhibiting effect of lysates is limited to the bursting of the streptococci by the phage, and that this process does not inactivate the tumour inhibiting factor There are no experimental results suggesting that phage is necessary for the tumour-inhibiting effect of the streptococci

It is difficult to determine whether the phage infection *could* nevertheless be a prerequisite for the tumour inhibiting effect The streptococcal strain used has been shown to be lysogenic (or perhaps a "carrier strain" (9)) All cultures used in the previous or present experiments to produce lysate, extract or filtrate from strain 3465, have therefore contained the phage produced by this strain It would hardly be possible to find a streptococcal strain that was definitely not lysogenic, as almost all bacterial strains examined for lysogenicity have been found to be lysogenic, whenever the investigation has been intensive enough (6) The possibility that the infectious phage particles are important for the inhibition of tumour can be excluded, for that reason only that the extracts were tumour inhibiting without containing *demonstrable* infectious phage particles However, the possibility cannot be excluded that changes in the bacterial metabolism as a result of phage or prophage (9 5) might be responsible for the emergence of the tumour inhibiting factor

However, there is one observation which strongly refutes the hypothesis that the production of tumour-inhibiting factor should be dependent on a definite phage or prophage A lysate of a haemolytic streptococcus belonging to Group C appears to be able to inhibit Brown-Pearce carcinoma (3) It seems unlikely that a Group C streptococcus could produce the same phage as a Group A streptococcus In order, therefore to maintain the hypothesis of a relationship between phage and tumour inhibiting effect, it would be necessary to assume that the decisive factor was the presence of a phage or prophage in the bacterium and that the effect of the phage or prophage was in itself nonspecific As mentioned however, there is no experimental basis for such a hypothesis

Using Koshimura's in vitro in vivo technique *Ohta* (11) divided nine strains of Group A [illegible] strains with stron[illegible] and one strain wi[illegible] cinoma A study [illegible] strains for lysogenicity might possibly be suggestive to the problem discussed

SUMMARY

Bacteria-free filtrate prepared from a strain of *Streptococcus pyogenes* has no inhibitory effect on Brown-Pearce carcinoma in young rabbits. This result is discussed in relation to the observation of a tumour-inhibiting effect of preparations of the same streptococcal strain, when these preparations are either 1) extracts of mechanically ground bacteria, or 2) lysates of bacteria, prepared by means of streptococcal bacteriophage.

The number of infectious phage particles in the various preparations is not related to the inhibitory effect on Brown-Pearce carcinoma.

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DEPARTMENT OF MEDICAL CHEMISTRY UNIVERSITY OF TURKU
TURKU 3 FINLAND

IN VITRO EXPERIMENTS WITH GRANULATION TISSUE ON THE MATURATION OF COLLAGEN

By

TAINA MAJANIEMI and E. KULONEN

Received 15 viii 63

Two stages can be defined in the formation of insoluble collagen fibres from tropocollagen macromolecules (1) the alignment of the tropocollagens which usually is accompanied by precipitation and (2) the maturation of the precipitated collagen into insoluble fibres Much attention has been paid to the mechanism of the first phase (*Schmitt* 1959) but the second is almost unknown even if much work has been devoted to the intermolecular cross links of collagen (for general reference for example *de la Burde Peckham & Veis* 1963) It is quite established that during the development of the collagenous tissue the soluble collagen is gradually converted to less soluble forms and that during the involution the reverse order applies

The purpose of this work was to explore the possibilities to study the insolubilization of collagen *in vitro* The method was to incubate *in vitro* various preparations of granulation tissue and analyze the solubility fractions on hydroxyproline Two kinds of experiments were made (1) without the addition of collagen and the results refer to endogenous collagen and (2) with additions of soluble collagen from the same species either in clear solution or in suspension of precipitated collagen where the tropocollagen macromolecules were in proper register already

EXPERIMENTAL

Preparation of the soluble collagens The procedure was modified from the paper by *Gross* (1958a) The skins of 10 guinea pigs (weighing 193–365 g) were [illegible] together 220 g) The hair [illegible]
was first minced with mea[illegible]
into 500 ml of 0 45 M NaCl [illegible]

This work is a part of a program supported by institutional grants from the [illegible] Sigrid Jusélius Foundation and U S *Department of Agriculture* Fore[illegible]
and Technical Program [illegible]
We are [illegible]
se pv [illegible]
c ntr l of [illegible]
M er [illegible]
our disp[illegible]

The supernatant was obtained by centrifugation (for 60 min at 50 000×g) and filtration through No 2 sintered 'Jena' glass filter and finally dialyzed overnight against pH 7 6 phosphate buffer (ionic strength = 0 4) Trichloroacetic acid was added (as 25 per cent solution w/v, in 0 45 M NaCl) in small portions until the solution contained about 2 5 per cent trichloroacetic acid and the pH was 3 5 The mixture was left to stand for 30 min and then centrifuged The supernatant was dialyzed against the same phosphate buffer, and the fluid remained clear The soluble collagen was precipitated with absolute ethanol, which was added slowly to the concentration of 14 per cent (v/v) The mixture was centrifuged after 24 hrs The precipitate was dissolved into 40 ml of 5 per cent acetic acid All the manipulations were performed at +4° C The final solution contained hydroxyproline about 5 mg/ml or about 3 5 per cent of collagen

For the experiments 1 4 ml of this solution in acetic acid was diluted with 3 6 ml of the Krebs 4A-solution (*Green & Lowther 1959*), and dialyzed for 72 hrs at +2° C against 100 ml of the same Krebs 4A salt solution containing 22 4 mM glucose The outside fluid was changed daily The precipitate which contained about the half of the collagen was removed by centrifugation (4000 r p m 30 min in cold room) and the clear supernatant was used in the experiments

To check whether the alignment of the tropocollagen macromolecules was significant for the action of the granuloma homogenate, acid soluble collagen of guinea pig skin (prepared by Dr *J Pikkarainen*) was precipitated by dialysis against 20 fold volume of Krebs 4A glucose solution which was changed daily for 4 days in the cold room The precipitate was suspended into 100 fold volume (v/wet weight) of the same Krebs 4A glucose solution and this suspension was used instead of collagen solutions With electron microscopy it was ascertained that the collagen was present in clean fibers with the normal periodicity

Experiments with carrageenin granuloma homogenates Commercial samples of carrageenin were used as 1–1 5 per cent (w/v) aqueous solution for the production of the granulation tissue in the guinea pig (average weight 410 g) Five ml of the carrageenin solution was injected subcutaneously in the abdominal flanks and the granulomata were harvested after several time intervals If not stated otherwise seven day old granulomata were used

The granulomata (15–40 g) were dissected and homogenized with an equal amount (v/wet weight) of the aforementioned Krebs 4A glucose solution, first with a Buhler homogenizer (3 min with full speed nominally 50 000 r p m cooled with water at +7° C) and finally with Potter-Elvehjem homogenizer (A H Thomas Cat Size C), by passing the teflon pestle six times to the bottom Nothing was removed from the homogenate which as a rule was used immediately For each batch of homogenate the granulation tissue was pooled from two guinea pigs and the respective control experiments were always carried out with the same homogenate

For the incubation 2 ml portions of the homogenate were taken The exogenous soluble collagen was added either as a clear solution or as a suspension of fibrous precipitate both in Krebs 4A glucose solution The amounts of added collagen were 5 mg (in 1 ml of solution) or 10 mg (in 1 ml of suspension) which had been found suitable in the preliminary experiments

The suspensions of homogenate with or without added collagen were incubated at + 37° C for various time intervals in Gallenkamp Shaking Incubator (No 6990) in erlenmeyer flasks and the controls were kept in cold room (+6° C) After incubation the flasks were chilled in ice water and stored in the cold room for several hours The contents of the flasks were homogenized with Potter Elvehjem homogenizer (A H Thomas size A nominal speed 2000 r p m) by passing the pestle six times to the bottom At this stage C 8 ml of Krebs 4A glucose solution was added also to the non incubated controls which were not homogenized at this stage

Experiments with other preparations of granulation tissue In preliminary experiments the granulomata were induced with viscose cellulose sponge (*Viljanto & Kulonen 1962*) They are not presented in detail because the carrageenin granulomata were preferred From the latter the preparation of the homogenates was easier and the granulomata contained larger fraction of soluble collagen even if the production of the granulomata was more variable

In the earlier phase some experiments were performed also with granuloma slices which were cut with a thin knife at 0 3 mm thickness Also this form of preparation was abandoned because the homogenate could be measured in more reproducible amounts

Fractionation of the homogenates The supernatant was obtained by centrifugation for 30 min at 3000 r.p.m. in the cold room. The sediment was rehomogenized with Potter-Elvehjem homogenizer as above into 6–10 ml of the desired solvent (salt or acetic acid solution). The homogenates were kept in the cold room overnight and then centrifuged as above. A control experiment was carried out to check the effect of a more powerful centrifugation. At 50 000×g about 2.9 26 per cent ($n = 6$) of the collagen left in the 0.45 *M* NaCl soluble fraction at the regular centrifugation was sedimented. However, the proportion was remarkably constant and therefore the significance of the results does not seem affected. The sediment from the acetic acid soluble fraction was negligible (about 4 per cent). In the series

centrifugation was carried out

drolyzed in sealed tubes in 6 N

xyproline was analyzed by the

licate. The standard curve was checked when new reagents had been prepared at intervals of about two weeks.

The amount of the total collagen varied even in parallel samples because of the difficulty in measuring the homogenate, and the following way of presentation was adopted. From the usual three parallel incubations absolute average amounts for each fraction were calculated. From the sum of these averages a correction coefficient was calculated to make the data of incubated samples comparable with the respective non-incubated samples.

RESULTS

Changes in the endogenous collagen of the granulation tissue. The proportional amounts of the various collagen fractions during the development of the granulomata are collected in Table 1. The peak of the collagen content is reached on the 10th day. The proportions of the fractions are rather constant except on the 4th day, when the amount of collagen is still very small. The amount of insoluble collagen never exceeds three fourths. The findings are in reasonable agreement with the study by *D. S. Jackson* (1957), who observed the peak of collagen content on the 14th day. According to his studies the proportion of the acid soluble fraction increased towards the involution of the granuloma.

The changes in the soluble hydroxyproline-containing fractions at incubation of the homogenates are shown in Table 2. The homogenate of 15 days-old granulation tissue differed from the general pattern. In spite of the large variation between the individual experiments, it seems justified to state that the soluble collagen decreased at the incubation mainly in the fractions which are soluble into 0.45 *M* NaCl and into 3 per cent acetic acid. The fraction which was soluble in Krebs 4A solution did not change so much, except in the case of 15 day old granuloma where it increased at the expense of the other soluble fractions and the total soluble fraction did not decrease at all during the incubation. The prolonged incubation for 4 hrs. did not obviously change the result of one hr. incubation.

Experiments were carried out also with homogenates stored for 7 and 13 days. In both experiments especially the 0.45 *M* NaCl soluble and acetic acid soluble fractions decreased, but the results were irregular because the amount of the insoluble collagen decreased in the case of 7 days old granuloma.

The supernatant was obtained by centrifugation (for 60 min at 50 000×g) and filtration through No 2 sintered Jena glass filter and finally dialyzed overnight against pH 7 6 phosphate buffer (ionic strength = 0 4) Trichloroacetic acid was added (as 25 per cent solution w/v, in 0 45 M NaCl) in small portions until the solution contained about 2 5 per cent trichloroacetic acid and the pH was 3 5 The mixture was left to stand for 30 min and then centrifuged The supernatant was dialyzed against the same phosphate buffer, and the fluid remained clear The soluble collagen was precipitated with absolute ethanol which was added slowly to the concentration of 14 per cent (v/v) The mixture was centrifuged after 24 hrs The precipitate was dissolved into 40 ml of 5 per cent acetic acid All the manipulations were performed at +4° C The final solution contained hydroxyproline about 5 mg/ml or about 3 5 per cent of collagen

For the experiments 1 4 ml of this solution in acetic acid was diluted with 3 6 ml of the Krebs 4A-solution (*Green & Lowther* 1959), and dialyzed for 72 hrs at +2° C against 100 ml of the same Krebs 4A salt solution containing 22 4 mM glucose The outside fluid was changed daily The precipitate, which contained about the half of the collagen was removed by centrifugation (4000 r p m , 30 min in cold room) and the clear supernatant was used in the experiments

To check whether the alignment of the tropocollagen macromolecules was significant for the action of the granuloma homogenate acid soluble collagen of guinea pig skin (prepared by Dr *J Pikkarainen*) was precipitated by dialysis against 20 fold volume of Krebs 4A glucose solution which was changed daily for 4 days in the cold room The precipitate was suspended into 100 fold volume (v/wet weight) of the same Krebs 4A glucose solution and this suspension was used instead of collagen solutions With electron microscopy it was ascertained that the collagen was present in clean fibers with the normal periodicity

Experiments with carrageenin granuloma homogenates Commercial samples of carrageenin were used as 1 1 5 per cent (w/v) aqueous solution for the production of the granulation tissue in the guinea pig (average weight 410 g) Five ml of the carrageenin solution was injected subcutaneously in the abdominal flanks and the granulomata were harvested after several time intervals If not stated otherwise seven day old granulomata were used

The granulomata (15–40 g) were dissected and homogenized with an equal amount (v/wet weight) of the aforementioned Krebs 4A glucose solution first with a Buhler homogenizer (3 min with full speed nominally 50 000 r p m cooled with water at +7° C) and finally with Potter Elvehjem homogenizer (A H Thomas Cat Size C) by passing the teflon pestle six times to the bottom Nothing was removed from the homogenate which as a rule was used immediately For each batch of homogenate the granulation tissue was pooled from two guinea pigs and the respective control experiments were always carried out with the same homogenate

For the incubation 2 ml portions of the homogenate were taken The exogenous soluble collagen was added either as a clear solution or as a suspension of fibrous precipitate both in Krebs 4A glucose solution The amounts of added collagen were 5 mg (in 1 ml of solution) or 10 mg (in 1 ml of suspension) which had been found suitable in the preliminary experiments

The suspensions of homogenate with or without added collagen were incubated at + 37° C for various time intervals in Gallenkamp Shaking Incubator (No 6990) in erlenmeyer flasks and the controls were kept in cold room (+6° C) After incubation the flasks were chilled in ice water and stored in the cold room for several hours The contents of the flasks were homogenized with Potter Elvehjem homogenizer (A H Thomas size A nominal speed 2000 r p m) by passing the pestle six times to the bottom At this stage 6 8 ml of Krebs 4A glucose solution was added also to the non incubated controls which were not homogenized at this stage

Experiments with other preparations of granulation tissue In preliminary experiments the granulomata were induced with viscose cellulose sponge (*Viljanto & Kulonen* 1962) They are not presented in detail because the carrageenin granulomata were preferred From the latter the preparation of the homogenates was easier and the granulomata contained larger fraction of soluble collagen even if the production of the granulomata was more variable

In the earlier phase some experiments were performed also with granuloma slices which were cut with a thin knife at 0 3 mm thickness Also this form of preparation was abandoned because the homogenate could be measured in more reproducible amounts

Fractionation of the homogenates The supernatant was obtained by centrifugation for 30 min at 3000 r p m in the cold room The sediment was rehomogenized with Potter Elvehjem homogenizer as above into 6–10 ml of the desired solvent (salt or acetic acid solution) The homogenates were kept in the cold room overnight and then centrifuged as above A control experiment was carried out to check the effect of a more powerful centrifugation At 50 000×g about 22 26 per cent ($n = 6$) of the collagen left in the 0 45 *M* NaCl soluble fraction at the regular centrifugation was sedimented However the proportion was remarkably constant and therefore the significance of the results does not seem affected The sediment from the acetic acid soluble fraction was negligible (about 4 per cent) In the series in which precipitated collagen had been added the centrifugation was carried out with a refrigerated MSE centrifuge (9000×g)

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difficulty in measuring the homogenate and the following way of presentation was adopted From the usual three parallel incubations absolute average amounts for each fraction were calculated From the sum of these averages a correction coefficient was calculated to make the data of incubated samples comparable with the respective non incubated samples

RESULTS

Changes in the endogenous collagen of the granulation tissue The proportional amounts of the various collagen fractions during the development of the granulomata are collected in Table 1 The peak of the collagen content is reached on the 10th day The proportions of the fractions are rather constant, except on the 4th day, when the amount of collagen is still very small The amount of insoluble collagen never exceeds three fourths The findings are in reasonable agreement with the study by *D S Jackson* (1957), who observed the peak of collagen content on the 14th day According to his studies the proportion of the acid soluble fraction increased towards the involution of the granuloma

The changes in the soluble hydroxyproline containing fractions at incubation of the homogenates are shown in Table 2 The homogenate of 15 day-old granulation tissue differed from the general pattern In spite of the large variation between the individual experiments, it seems justified to state that the soluble collagen decreased at the incubation mainly in the fractions which are soluble into 0 45 *M* NaCl and into 3 per cent acetic acid The fraction which was soluble in Krebs 4 A solution did not change so much except in the case of 15 day old granuloma where it increased at the expense of the other soluble fractions and the total soluble fraction did not decrease at all during the incubation The prolonged incubation for 4 hrs did not obviously change the result of one hr incubation

Experiments were carried out also with homogenates stored for 7 and 13 days In both experiments especially the 0 45 *M* NaCl soluble and acetic acid soluble fractions decreased but the results were irregular, because the amount of the insoluble collagen decreased in the case of 7 days old granuloma

TABLE 1

The Hydroxyproline Containing Fractions of Carrageenin Granulomata during the Development

Age of the granulation tissue	Total hydroxyproline μg	Fraction of hydroxyproline %			
		Soluble in Krebs 4A solution	Soluble in 0.4 M NaCl	Soluble in 3% acetic acid	Insoluble
4 days	155	37	14	9	40
6 days A	612	15	8	10	67
6 days B	1210	11	12	11	66
7 days	1410	10	8	8	74
10 days	1640	10	8	8	74
15 days	1200	11	12	9	68

The figures are derived from the averages of duplicate or triplicate determinations

TABLE 2

Changes of the Soluble Hydroxyproline-Containing Fractions of Granulation Tissue at Incubation

Age of the granulation tissue	Type of preparation	Total soluble hydroxyproline initially μg	Change in μg			
			Total	In the fractions soluble in		
				Krebs 4A solution	0.45 M NaCl solution	3% acetic acid
4 days	homog	93	−10	−3	−4	−3
6 days A	homog	200	−37	−4	−20	−13
6 days B	homog	420	−35	−2	−16	−17
7 days A	homog	360	−9	+7	−21	+5
7 days B	homog	80	−20	−9	−7	−4
10 days	homog	430	−50	+2	−17	−35
15 days	homog	380	+3	+33	−8	−22
7 days C	slices	190	−26	−19	−3	−4
6 days C	homog	120	−30	0	−2	−28
6 days C *	homog	120	−28	0	−4	−24

* incubated for 4 hrs

Duration of incubation 1 hr. Figures are derived from averages of triplicate experiments. No exogenous collagen was added.

Two series of experiments were carried out in the presence of β-aminopropionitrile (0.01 M). The first two experiments were made with homogenates of 6 and 7 days old granulomata. In short, they indicated that the presence of β-aminopropionitrile hydrosulfate prevented the effect of incubation. However, when the experiment was repeated with 0.001 M β-aminopropionitrile and the hydrosulfate was carefully neutralized, no effect of the presence of β-aminopropionitrile was observed.

Changes in added collagen. From Table 3 it is evident that the presence of the homogenate during the incubation induced an increase in the NaCl and acetic acid soluble fractions of added collagen, even if the collagen had been added as a precipitated suspension.

TABLE 3

Effect of Incubation of the Granulation Tissue on Added Precipitated Collagen

Solubility	Incubated						Not incubated
	With homogenate			Without homogenate			
	Exp I	Exp II	Average	Exp I	Exp II	Average	
Krebs 4A solution	58.7	74.3	*66.5*	56.9	65.3	*61.2*	*18.4*
0.45 M NaCl	8.9	5.7	*7.3*	0.6	0.9	*0.7*	*1.6*
3% acetic acid	19.0	9.0	*14.0*	13.0	5.4	*9.2*	*19.5*
Insoluble	13.5	11.0	*12.2*	29.5	28.4	*28.9*	*60.4*

The experimental figures are in per cent of the total hydroxyproline Two independent sets of experiments (designated I and II) were carried out The figures are derived from the averages of three incubation experiments Time of incubation 1 hr

Especially the salt soluble fraction was increased The incubation itself caused a large increase of the Krebs 4A soluble supernatant fraction presumably because of denaturation of the collagen This por

ogenate, but the insoluble

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fraction is in the non dif

fusible form The proportion of the diffusible hydroxyproline was not affected by the presence of homogenate

Several experiments were made with the addition of the soluble col *lagen* as a solution in Krebs 4A glucose Here will be presented only those which were made in triplicate (Table 4) From the data it is evident that also in this case the presence of the homogenate enhanced the proportional amount the fraction soluble in 0 45 M NaCl, and decreased that which is soluble in Krebs 4A solution In the less controlled *experiments the increase in the salt soluble fraction* was also found as a rule

TABLE 4

Effect of Incubation of the Granulation Tissue on Added Collagen Solution

Solubility	Incubated							
	With granulation tissue				Without granulation tissue			
	Exp I	Exp II	Exp III	Average	Exp I	Exp II	Exp III	Average
Krebs 4A solution	34.7	13.1	15.4	*21.1*	40.4	30.1	28.9	*33.1*
0.45 M NaCl	58.3	80.0	41.3	*59.8*	40.9	60.8	34.0	*45.2*
3 acetic acid	5.9	6.8	26.3	*13.0*	16.7	4.8	32.7	*18.1*
Insoluble	1.0		16.9	*6.0*	2.1	5.3	4.6	*4.0*

The figures are in per cent of the total hydroxyproline Three independent sets of experiments were made I and II with sponge granuloma homogenate III with carrageenin granuloma homogenate *The figures are averages of three incubation* experiments Time of incubation 1 hr

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The figures are derived from the averages of duplicate or triplicate determinations

TABLE 2

Changes of the Soluble Hydroxyproline Containing Fractions of Granulation Tissue at Incubation

Age of the granulation tissue	Type of preparation	Total soluble hydroxyproline initially µg	Change in µg			
			Total	In the fractions soluble in		
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6 days B	homog	420	—35	—2	—16	—17
7 days A	homog	360	—9	+7	—21	+5
7 days B	homog	80	—20	—9	—7	—4
10 days	homog	430	—50	+2	—17	—35
15 days	homog	380	+3	+33	—8	—22
7 days C	slices	190	—26	—19	—3	—4
6 days C	homog	120	—30	0	—2	—28
6 days C*	homog	120	—28	0	—4	—24

* incubated for 4 hrs
Duration of incubation 1 hr Figures are derived from averages of triplicate experiments No exogenous collagen was added

Two series of experiments were carried out in the presence of β-amino propionitrile (0.01 M) The first two experiments were made with homogenates of 6 and 7 days old granulomata In short, they indicated that the presence of β-aminopropionitrile hydrosulfate prevented the effect of incubation However, when the experiment was repeated with 0.001 M β aminopropionitrile and the hydrosulfate was carefully neutralized, no effect of the presence of β aminopropionitrile was observed

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Effect of Incubation of the Granulation Tissue on Added Precipitated Collagen

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0.45 M NaCl „	8.9	5.7	*7.3*	0.6	0.9	*0.7*	*1.6*
3% acetic acid	19.0	9.0	*14.0*	13.0	5.4	*9.2*	*19.5*
Insoluble	13.5	11.0	*12.2*	29.5	28.4	*28.9*	*60.4*

The experimental figures are in per cent of the total hydroxyproline. Two independent sets of experiments (designated I and II) were carried out. The figures are derived from the averages of three incubation experiments. Time of incubation 1 hr.

Especially the salt soluble fraction was increased. The incubation itself caused a large increase of the "Krebs 4A-soluble" supernatant fraction presumably because of denaturation of the collagen. This portion was not largely influenced by the homogenate, but the insoluble fraction decreased markedly, if homogenate had been present. Most of the hydroxyproline in the Krebs 4A-soluble fraction is in the non diffusible form. The proportion of the diffusible hydroxyproline was not affected by the presence of homogenate.

Several experiments were made with the addition of the soluble collagen as a solution in Krebs 4A glucose. Here will be presented only those which were made in triplicate (Table 4). From the data it is evident, that also in this case, the presence of the homogenate enhanced the proportional amount the fraction, soluble in 0.45 M NaCl, and decreased that which is soluble in Krebs 4A solution. In the less controlled experiments the increase in the salt soluble fraction was also found, as a rule.

TABLE 4

Effect of Incubation of the Granulation Tissue on Added Collagen Solution

Solubility	Incubated							
	With granulation tissue				Without granulation tissue			
	Exp. I	Exp. II	Exp. III	Average	Exp. I	Exp. II	Exp. III	Average
Krebs 4A solution	34.7	13.1	15.4	*21.1*	40.4	30.1	28.9	*33.1*
0.45 M NaCl	58.3	80.0	41.3	*59.8*	40.9	60.8	34.0	*45.2*
3% acetic acid	5.9	6.8	26.3	*13.0*	16.7	4.8	32.7	*18.1*
Insoluble	1.0		16.9	*6.0*	2.1	5.3	4.6	*4.0*

The figures are in per cent of the total hydroxyproline. Three independent sets of experiments were made: I and II with sponge granuloma homogenate, III with carrageenin granuloma homogenate. The figures are averages of three incubation experiments. Time of incubation 1 hr.

DISCUSSION

Nature of the changes These experiments have some bearing on the work of *Gross* (1958b) He studied the reversibility of the heat gelation of various preparations of soluble collagen and found that especially the crude extracts were partly insoluble after heating for various periods He suggests "*that the steadily diminishing solubility of precipitated fibrils with time is, at least in part, a result of the interacting collagen molecules finding their most stable state of association*" It is also a common experience that the amount of soluble collagen decreases *in vitro* in many tissues with time and that the precipitates of soluble collagen are not always fully soluble The present experiments could be *interpreted in the same terms, but it should be noted that the decrease* in the solubility decreases to a certain limit only and that there seems to be a kind of equilibrium between the collagen fractions, which is especially manifested in Table 1

It remains to be investigated whether these changes are purely physicochemical, depending on the precipitation with non-collagenous materials or on improved alignment, or whether they involve enzymatic activity

It is obvious that part of the added collagen is denatured by the incubation, but not degraded to diffusible form (Table 3) The presence of homogenate induces the disintegration of the insoluble form to salt-soluble forms What happens in the terms of chemical bonds, is not known *Frankland & Wynn* (1962) and *Barsky & Garrison* (1963) have shown that liver, especially its microsomal fraction, contains an enzyme-like principle, which breaks down collagen, as has been demonstrated also by *Nagai, Lapière & Gross* (1963) with other tissues Homogenate may be assumed to contain something which penetrates between the collagen macromolecules and thus renders the fibres soluble

At incubation the added pure fibrous collagen and the endogenous collagen of the granuloma itself changed in different manner On this basis it may be further assumed that in the natural fibrillogenesis some non-collagenous matter is significant (cf *Partington & Wood* 1963) When the homogenates were prepared, the endogenous soluble collagen was not converted into solution at all, but retained the natural configuration *in situ*, which may be significant in the normal maturation

Are changes developing in the intra- or intermolecular linkages at incubation? As regards endogenous collagen the question is thus far impossible to settle experimentally, because the soluble collagen is only a very minor fraction of the accompanying soluble proteins and methods have first to be developed for the analysis of the subcomponents of collagen *in the extracts of granulation tissue*

The study of the changes in the added collagens is more readily practicable, but thus far the sedimentation analysis (by Dr *T Nikkari*) hat not provided other results than non-defined changes in the α and

β fractions during incubation with tissue homogenate The interpretation of the experiments is difficult also because the added collagen partly precipitates during the incubation at +37° C

SUMMARY

The changes in the solubility of collagen were studied by incubation experiments with granulation tissue preparations

The proportions of the collagen fractions (soluble in salt solutions or in acetic acid) were fairly constant during the growth of the carrageenin granulomata for up to 15 days

The soluble collagen fractions, which were present in the granuloma homogenate itself, decreased at the incubation The collagen, which had been added either in clear solution or in suspension of precipitate, behaved differently at the incubation with granuloma preparations The presence of the homogenate increased the salt-soluble fraction In addition, the insoluble fraction of the precipitated collagen decreased markedly

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FROM RHEUMATIC RESEARCH LABORATORY, INSTITUTE OF PATHOLOGICAL ANATOMY (HEAD PROFESSOR G. TEILUM, M.D.) AND INSTITUTE OF BIOCHEMISTRY (HEAD PROFESSOR F. LUNDQUIST PH.D.) UNIVERSITY OF COPENHAGEN, DENMARK

STUDIES ON A TRANSPLANTABLE MASTOCYTOMA IN MICE

3. *Histologic and Ultrastructural Changes Following Varying Doses of Oestrogen*

By

O. H. Iversen, H. E. Christensen and R. Rask-Nielsen

Received 1.viii.63

The influence excerted by oestrogen hormones on the tissue mast cells has been the subject of several investigations. Thus *Kelsall & Crabb* (1956) in female hamsters after treatment with oestrogen hormones found increased numbers of mast cells, increased content of acid mucopolysaccharides and of collagen interstitially in mammae. They further observed, that smaller doses did not cause changes in the number of mast cells of the myometrium, whereas larger doses were followed by increased numbers of mast cells. *Johansson & Westin* (1959) observed that a single injection of oestrogen hormone to oophorectomized mice 24 hours later had resulted in a decrease of the number of mast cells in the uterine horns. *Westin & Odeblad* (1956) correspondingly found in mice vaginae decreased numbers of mast cells after oestrogen treatment; qualitatively, however, the cells were heavily metachromatic without distinct granules. *Iversen* (1962) investigated the effect of oestrogenic treatment on the mast cells in uteri of oophorectomized guinea pigs; 0.25 mgs every second day, in total 4 injections or 1.0 mg, caused decreased numbers of mast cells, and morphologically a degranulation of the cells with appearance of a distinct metachromatic halo around them was observed. Following doses of 0.5 mg daily for 14 days, however, an increase of the number of mast cells was found; morphologically the cells were more intensely metachromatic stained and in general larger; only few cells showed metachromatic halo. *Iversen & Christensen* (1963) investigated the changes in mast cells of the uteri of pregnant guinea pigs. Early in pregnancy no changes in the mast cell population were found; the cells contained distinct orthochromatic granules. At the end of pregnancy however the number of mast cells increased to a considerable extent, the cells were larger, but the mast cell granules were less distinct and often vacuoles and metachromatic halo were present. These changes are possibly related to some increased oestrogen release towards the end of pregnancy.

Previously two transplantable mastocytomas in mice have been described *Dunn & Potter* (1957) and *Furth, Hagen & Hirsch* (1957) Electron microscopic studies on these mastocytomas were carried out by *Hagen Barrnett & Lee* (1959) *Mengel & Trier* (1961) and *Bloom* (1960) *Rask Nielsen & Christensen* (1963) have given a report of the origin and general morphology of a third transplantable mastocytoma in mice The primary tumour arose in a 26 months old female (CBA × DBA 2) F_1 mouse which at the age of 4 months had been inoculated with leukaemic tissue from a transplantable plasma cell leukaemia but without signs of growth from that tumour The investigations were carried out on mice from the first and the second passages of the transplantation of the mastocytoma This tumour showed slow growth rate and the cells much resembled normal mast cells, thus containing numerous metachromatic and orthochromatic granules often covering the cell nucleus, histochemically the granules were found to contain acid mucopolysaccharides *Christensen, Iversen & Rask Nielsen* (1963) investigated the ultrastructure of this highly differentiated mastocytoma and found the following characteristic data The Golgi region was well developed Free ribosomes and ribosomes attached to membranes and a well developed endoplasmic reticulum were frequently found in the cytoplasm The mast cell granules were lodged in lacunes, most often in the outer part of the cytoplasm The structure of the granules varied from loose structured to homogen and dense granules From the cell surface elongated, slender villi projected

On the basis of these above mentioned observations and those of others *Christensen, Iversen & Rask Nielsen* (1963) have put forward the following hypothesis about the synthesis of the mast cell granules A direct connection leads from the interior of the tumour mast cell via the channels of the endoplasmic reticulum to the lacunes in the outer part of the cytoplasm where secreted material is built up to form the granules From the lacunes the mast cell granules are released to the extracellular space and during this process the granules may be dissolved

The present paper is a report on investigations in which the effects of oestrogenic treatment on the transplanted tumours and the mastocytoma cells was studied

MATERIAL AND METHODS

5 m ce transplanted subcutaneously with the mastocytoma of *Rask Nielsen &* Chr stensen (1963) were used for the investigations In all cases biopsy of the tumour was taken hef re ho [illegible] of peanut oil [illegible] injections dissolved in pe [illegible] ovex Leo ®

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FROM RHEUMATIC RESEARCH LABORATORY, INSTITUTE OF PATHOLOGICAL ANATOMY (HEAD PROFESSOR G TEILUM, M D) AND INSTITUTE OF BIOCHEMISTRY (HEAD PROFESSOR F. LUNDQUIST, PH D) UNIVERSITY OF COPENHAGEN, DENMARK

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MATERIAL AND METHODS

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For *histologisk* investig
per cent ethanol (100 cc) 10 cc formalin and 5 cc acetic acid ... ixture of 70
The following staining methods were used Toluidine blue pH 4 5 and 6 5 alcian blue safranine (*Spicer* 1960) alcian blue (*Wagner & Shapiro* 1957) azur A pH 4 0

FROM RHEUMATIC RESEARCH LABORATORY, INSTITUTE OF PATHOLOGICAL ANATOMY (HEAD PROFESSOR G TEILUM, M D) AND INSTITUTE OF BIOCHEMISTRY (HEAD PROFESSOR F LUNDQUIST, PH D) UNIVERSITY OF COPENHAGEN, DENMARK

STUDIES ON A TRANSPLANTABLE MASTOCYTOMA IN MICE

3 *Histologic and Ultrastructural Changes Following Varying Doses of Oestrogen*

By

O. H Iversen, H E Christensen and R Rask-Nielsen

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The present paper is a report on investigations, in which the effects of oestrogenic treatment on the transplanted tumours and the mastocytoma cells was studied

MATERIAL AND METHODS

5 mice transplanted subcutaneously with the mastocytoma of *Rask Nielsen & Christensen* (1963) were used for the investigations In all cases biopsy of the tumour was taken before the experiment started Two mice were given injections of peanut oil while three mice received injections of oestrogen (Diprovex Leo ® dissolved in peanut oil) The mode of treatment is visualized in Table 1

For histologisk investigation the t… per cen… …ture of 70

bl… 6 5 alcian A pH 4 0

Astral lau (*Bloom & Kelly* 1960) methylgreen pyronine and PAS technique In cul ation with ribonuclease was used to control the specificity of the methylgreen pyronine staining

TABLE 1

Mode of treatment						
	Mouse no	1 biopsy	treatment	2 biopsy	treatment	3 biopsy
Group I (control group)	4211	1 day	0 1 cc peanut oil subc	14 day	0 1 cc peanut oil subc	28 day
	4642	1 day	0 1 cc peanut oil subc	14 day	0 1 cc peanut oil subc	28 day
Group II (oestrogen treated group)	4299	1 day	25 γ oestrogen × 6 subc	14 day	50 γ oestrogen × 6 subc	28 day
	4612	1 day	25 γ oestrogen × 6 subc	14 day	25 γ oestrogen × 6 subc	28 day
	4676	1 day	25 γ oestrogen × 6 subc	14 day	25 γ oestrogen × 6 subc	28 day

For *electron microscopy* the biopsies were promptly cut into small tissue blocks measuring 1 mm³ and fixed for two hours in buffered osmiumtetroxide (pH 7.2) Dehydration and embedding in Vestopal W (Martin Jaeger Switzerland) was done after *Zelickson & Hartmann* (1960) Thin sections were cut in a LKB ultramicrotome After staining of sections for 1 2 hours with a 0 5 per cent solution of uranyl acetate was used A RCA (FMU 2F) electron microscope was used

OBSERVATIONS

Light microscopy The histologic appearance of the untreated mastocytoma was previously described by *Rasl Nielsen & Christensen* (1963) In the present work the control groups showed a corresponding picture It shall be mentioned however that the metachromasia with toluidine blue was more pronounced at pH 6 5 than at pH 4 5

After oestrogen treatment some changes occurred In toluidine blue azur A and Astrablau stainings the 2nd and 3rd biopsies showed numerous mast cell granules extracellularly but metachromatic halo around the cells was rare In alcian blue safranine staining more cells with alcian blue positive granules were found after oestrogen treatment than in the control groups and in many cells the peripheral location of the granules after oestrogen treatment was demonstrated so that the cells centrally appeared red and peripherally blue (alcian blue) Further oestrogen treatment did cause a rise in the number of pyroninophilic cells as well as an augmentation of the staining intensity with this dye The pyroninophilia was sensitive for ribonuclease indicating the presence of ribonucleic acid which is in accordance with the findings of increased numbers of ribosomes by electron microscopy PAS technique did not reveal any significant changes the cells in the control groups and in the treated groups were faintly stained



Fig 1

Tumour mast cell from control mouse The cell nucleus is large with a central position chromatine is found beneath the nuclear membrane and in spots in the interior Perinuclearly endoplasmic reticulum and mitochondries are found The mast cell granules are diffusely spread in the cytoplasm but mostly peripherally in the cell The granules show different structures most of them are loose structured while a few are homogenous The lacunes in which the mast cell granules are lodged are of varying size and form The cell surface with some cell surface protrusions are seen in the upper corners and lower left corner × 22 100

Electron microscopy By electron-microscopy the tumour mast cells of the oestrogen treated mice showed some ultrastructural alterations, which quantitatively and qualitatively differed from the described ultrastructure of the mast cell neoplasm (*Christensen, Iversen & Rask-Nielsen* 1963) and also from the control material used in this study

Astrablau (*Bloom & Kelly* 1960), methylgreen pyronine and PAS technique. Incubation with ribonuclease was used to control the specificity of the methylgreen pyronine staining.

TABLE 1

	Mode of treatment					
	Mouse no.	1. biopsy	treatment	2. biopsy	treatment	3. biopsy
Group I (control group)	4211	1. day	0.1 cc peanut oil subc.	14. day	0.1 cc peanut oil subc.	28. day
	4642	1. day	0.1 cc peanut oil subc.	14. day	0.1 cc peanut oil subc.	28. day
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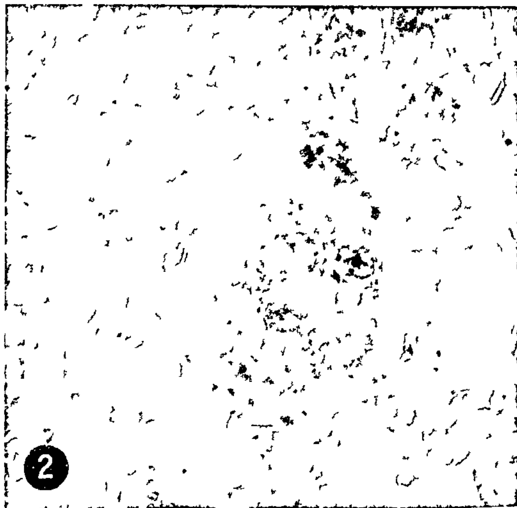
Fig 3

Mast cells and interstitium after oestrogen treatment 2nd biopsy The large inter cellular space with abundant collagen is visualized In the cell to the right the granules are situated just beneath the cell surface in large lacunes and showing beginning dissolution Long slender cell surface protrusions are seen × 22 100

The cell nucleus Oestrogen treatment apparently caused the accumulation of electron dense material in the nuclei especially beneath the double nuclear membrane (Fig 4)

The cytoplasm In principle the cytoplasm had the same appearance in oestrogen treated as in non treated animals Around the nucleus was an inner zone containing the Golgi region ribosomes, portions of the endoplasmic reticulum and mitochondries Peripherally in the cell were lacunae in which the mast cell granules were lodged

The Golgi region was well developed in the oestrogen treated tumours perhaps more so in the second biopsy than in the third As ordinarily it was located in an indentation of the nucleus and consisted of lamellar and canalicular systems, vesicules and fine granules



F g 2

Tum ur mast cells from control mouse of m re polymorphous character than the cell in F g 1 To the left from the cell nucle s in the centre a well developed Golg region is seen × 14 000

Figs 1 2 illustrate tumour mast cells from the control material The cells in the un treated animals were nearly of the same size as normal mast cells 8 20 μ and of polygoneal form with rounded corners

After hormonal treatment the cells were more widely separated from each other the interstitia being filled with a ground substance or oedema showing only little osmiophilia in many of these considerable amounts of collagen were seen (Fig 3) In addition the number of immature cells was lower than in non treated animals In general the oestrogen treatment was coincident with the presence of well developed cell organelles showing a high degree of organization But as mentioned quantitative and qualitative differences between treated and non treated animals were observed which is illustrated by the following more detailed description of the cell organelles



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Fig 4

Tumour mast cell after oestrogen treatment, 2nd biopsy The cell is polygonal
titium to the neighbouring
the granules are situated
in dissolution often lying
× 14 620

The mitochondria did not display any exceptional peculiarities

Ribosomes and endoplasmic reticulum These structures showed rather pronounced changes, when the animals had been treated with oestrogenic substance Especially in the second biopsy the cells contained a well-developed endoplasmic reticulum with numerous ribosomes The membranes of the endoplasmic reticulum formed a complex system of channels Most of the ribosomes were attached to membranes (Figs 5–6) There was a marked difference between these findings and the picture seen in the third biopsy, where the produced mast cell granules dominated, and the endoplasmic reticulum was now more poorly developed, forming straight channels and few ribosomes (Fig 7)

The lacunae located peripherally in the tumour mast cells were



Fig 5

Part of mast cell after oestrogen treatment A well developed endoplasmic reticulum with ribosomes is demonstrated The possible termination of the channels of the endoplasmic reticulum into the lacunes has been discussed by *Christensen Iversen & Rask Nielsen* (1963) Mast cell granules in lacunes are found peripherally in the cell × 32,300

numerous in the oestrogen treated animals They varied much in size, but often they were large and close to each other The lacunes were often covered only by the cell surface protrusions, so that the lacunae were actually invaginations of the outer cell membrane (Figs 3, 4 and 8) The mast cell granules did not fill out the whole space of the lacu nes the clear space around the granules often dominated, giving the structures a vesicular appearance In the third biopsy the lacunes might be found more diffusely spread in the cytoplasm, but still mostly in the outer part of the cytoplasm as known from the untreated animals (Fig 7)

The mast cell granules measured 0 5 1 0 μ and were numerous in all

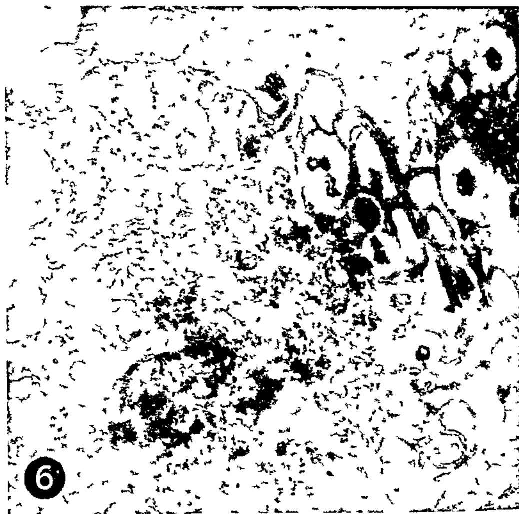


Fig 6

Character stic] icture from oestrogen treate l grou] ?n l biops\ W ell develo] e l endo] lasm c reticulum mast cell granules in dissolution in lacunes or covered only l\ the cell s irface] rotrus ons and interstitia \ itl collagen × 3? 300

biopsies but especially in the third bio] s\ in which the granules might be spread all over the cytoplasm (as above-mentioned for the lacunes in which the gr inules are lodged) Then the granules might be large and confluent The structure of the mast cell granules varied much and many transitional forms between loose structured and homogen gra nules were found But after oestrogen tre itment the granules of homo gen structure were relatively seldom seen In the] eriphery of many granules small ring sha] ed bodies with a more electron dense lamellar structure were seen (I ig 9) Often in the ?nd and 3rd biopsies these ring sh a] ed lamellar l odies did form a whole granulum I igs 10 11) but sometimes these structures were found free in a lacun i without any other osmiophil substance between them (Fig 10) These structural changes might be found in the n n oestrogen treated anim ils whereas they were pronounced in the oe trogen treated group

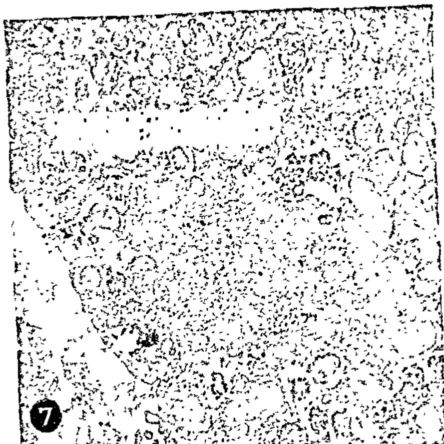


Fig 7.

Characteristic picture from oestrogen treated group, 3rd biopsy. The mast cell granules are numerous and diffusely spread in the cytoplasm, some are confluent, while others are vesicular appearance Only small portions of endoplasmic reticulum are now *found* × 22,100.

Disintegration and dissolution of granules was most pronounced in the 2nd biopsy. The granules then had a peripheral [illegible] and were [illegible] est of the [illegible] granules [illegible] 4 and 8).

[illegible] protrusions These structures showed a definite increase in number and size after *oestrogenic* treatment, especially in the 2nd biopsy. Indeed, these microvilli might [illegible]

[illegible] disintegrated [illegible] dissolved mast cell granules were seen (Fig. 8)

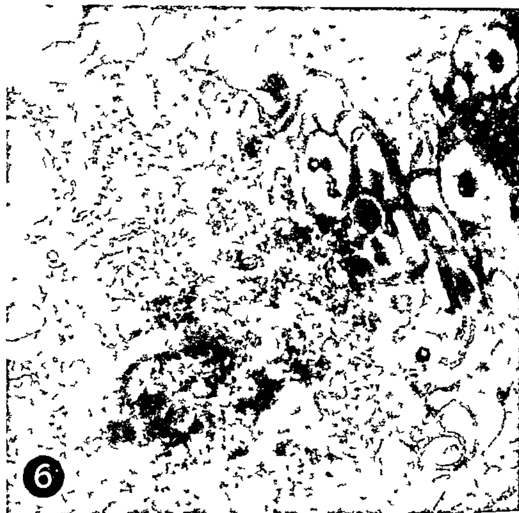


Fig. 6

Characteristic picture from oestrogen treated group, 2nd biopsy. Well developed endoplasmic reticulum, mast cell granules in dissolution in lacunae or covered only by the cell surface protrusions and interstitium with collagen. × 32 300

biopsies but especially in the third biopsy, in which the granules might be spread all over the cytoplasm (as above-mentioned for the lacunes in which the granules are lodged). Then the granules might be large and confluent. The structure of the mast cell granules varied much and many transitional forms between loose structured and homogen granules were found. But after oestrogen treatment the granules of homogen structure were relatively seldom seen. In the periphery of many granules small ring shaped bodies with a more electron dense lamellar structure were seen (Fig. 9). Often in the 2nd and 3rd biopsies these ring shaped lamellar bodies did form a whole granulum (Figs. 10, 11), but sometimes these structures were found free in a lacuna without any other osmiophil substance between them (Fig. 10). These structural changes might be found in the non oestrogen treated animals, whereas they were pronounced in the oestrogen treated group.



Fig 11

Part of tumour mast cell with granules after oestrogen treatment, 3rd biopsy The picture illustrates the change in the structure of the granules Many granules consist of circular lamelles × 32,300

DISCUSSION

The effect of oestrogenic treatment on the morphology of tumour mast cells has not previously been investigated But *Furth, Hagen & Hirsch* (1957) found, that such treatment macroscopically did not influence the growth rate of their mouse mastocytoma The same was true of our mastocytoma —*Asboe-Hansen* (1954) has investigated by light- and electron-microscopy the influence of cortisone on normal mast cells and found degranulation, halo and vacuolization, and by electron-microscopy (after freeze drying) a conglomeration of the granules in the cytoplasm was seen

The present investigations have shown that treatment with oestrogen hormone (especially after a shorter period of time *viz* 2nd biopsy) causes a more ample development of the synthetic and secretory apparatus of the tumour mast cell We found especially a well-developed Golgi region and an ample endoplasmic reticulum Gradually an increase in the number of mast cell granules was seen The granules were found in the periphery of the cell, more so than in the control material, and signs of excretion and dissolution of granules were rather often met with

These observations are in accordance with previous findings by light microscopy on mast cells in uteri of man and guinea pigs after oestrogen treatment *Iversen* (1960), *Iversen* (1962) and *Iversen & Christensen* (1963) They found a definite metachromatic halo around the cells and degranulation However, the phenomena of "halo" and "degranulation" have often been discussed Thus *Asboe-Hansen* (1954) and *Cavallero & Braccini* (1951) found cortisone to produce degranulation, *Riley & West* (1953) and *Fawcett* (1955) found degranulation and halo

Fig 8

Boundary line between four mast cells after oestrogen treatment 2nd biopsy The cell surface protrusions or microvilli are demonstrated The intercellular spaces are rather wide × 22 100



Figs 9–10

Fig 9 Part of tumour mast cell after oestrogen treatment 2nd biopsy Four large granules with a well developed membrane are seen Within the granules are circular or oval somewhat irregular lamellar formations which are more osmiophil than the otherwise homogenous granules × 22 100

Fig 10 Part of tumour mast cell with granules and lacunes after oestrogen treatment 2nd biopsy In many lacunes are circular bodies lying isolated without intervening osmiophil substance × 22 100

further extent than in the control groups The intercellular spaces be came filled with collagen after oestrogen treatment

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after histamin-liberators Furthermore degranulation has been described after benzol (*Sylven & Larsson* 1948) and snake venom (*Zahl & Novak* 1951) *Smith & Lewis* (1957) investigated, by electron-microscopy, the peritoneal mast cells of hamsters and found conglomeration and loose-structured granules after treatment with toluidine blue, protamine sulphate and stilbamine However, some investigators are of the opinion, that halo is not a morphologic criterium, but an artefact (*Devitt et al* 1954, *Padawer & Gordon* 1954)

The ring-shaped lamellar bodies (Figs 9–11) found in our mastocytoma as parts of mast cell granules or lying free in the lacunes are identical to such structures observed by *Hibbs, Burch & Phillips* (1960) in the human mast cell, and to intragranular cylindres described by *Orfanos & Stuttgen* (1962) after acute action of 48 80 in a skin biopsy from a child with congenital mastocytosis The latter investigators described different stages in the development of the granules, the cylindres should be the first stage in the secretion process, and thereafter followed different stages with the formation of condensation centers, loss of substance peripherally in the granules and eventually excretion of condensation centers from the cell

Our observations concerning the intragranular structure are also regarded as the intragranular response to the stimulation provoked on the cell by oestrogen treatment, and the different structural changes (Figs 9–11) are stages towards total disintegration and dissolution of the mast cell granules The observed histochemical changes after oestrogen treatment, *viz* increased alcian blue positivity on the expense of safranine positivity, also speaks in favour of such structural and chemical changes due to the compound

We have found that oestrogen treatment causes the tumour mast cell to increase its synthezising activities, especially the formation of granules, and causes release of granules, which are readily dissolved extracellularly *Rogers* (1956) who investigated the mast cells of the mouse cutis by electron microscopy also stated, that the mast cell granules are excreted from the cell, and was of the opinion, that the mast cells were able to form the ground substance in tissues, in which a synthesis of collagen took place

SUMMARY

The effects of oestrogen treatment on the tumour mast cells of a transplantable mastocytoma in mice were investigated Rather pronounced changes in the ultrastructure of the cells were observed Especially signs of increased synthetic activities of the Golgi region and the endoplasmic reticulum were found after smaller doses of oestrogen Numerous mast cell granules were formed Also the structure of the mast cell granules was altered, and it was striking that disintegration and dissolution and eventually release took place to a

further extent than in the control groups The intercellular spaces became filled with collagen after oestrogen treatment

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FROM THE MUNICIPAL VIROLOGICAL LABORATORY AND THE VIROLOGICAL LABORATORY OF THE DEPARTMENT OF BACTERIOLOGY UNIVERSITY OF GOTHENBURG SWEDEN

A TISSUE CULTURE METHOD FOR TITRATION OF INFECTIVITY AND DETERMINATION OF GROWTH RATE OF TOXOPLASMA GONDII I[1]

By

ERIK LYCKE and EBBA LUND

Received 21 vi 63

On the basis of observations in phase contrast microscopy (1, 2) of HeLa cell cultures infected with toxoplasma parasites a method has been worked out for titration of the infectivity of toxoplasma parasites In the present study the standardization of the parasite suspensions and the cell cultures is reported In a subsequent report (3) the method of titration is described and its accuracy assayed

MATERIAL AND METHODS

The parasites The RH strain of *Toxoplasma gondii* was used The strain was maintained by serial inoculations into six week old Swiss albino mice of our laboratory stock. *Parasite* suspensions were prepared from freshly harvested ...
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ring of stainless steel ... diameter (1 35 mm was fastened to the slides by means of warm beeswax The culture medium with 200 000 cells was added in a volume of 0 4 ml This cell number was found to be optimal all wing outgrowth of the cultures in two days On the top of the ring a cover glass was placed and sealed to the ring with warm vaseline The rings and the cover

The skillful technical assistance of *Miss Mona Torstensson* and *Miss Marie Louise Brodenfal* is gratefully acknowledged
[1] This investigation was supported by PHS research grant AI 05074 01 from *National Institute of Allergy and* ... *ious Diseases Public Health Service U S A*

glasses had been sterilized in dry heat The beeswax and the vaseline were used unsterilized

If not otherwise stated the tissue culture medium consisted of Hanks solution with additions of 0.5 per cent lactalbumin hydrolysate 20 per cent dye test negative human serum 100 I U of penicillin 100 gamma streptomycin and sodium bicarbonate to obtain a pH of 7.2 The cultures were incubated at 37° C for two days whereafter the cultures were inoculated with parasites and reincubated The cultures could then be maintained in an apparently good condition for a further 48 hours without changing the medium

Microscopy The readings of the cultures were made with a phase contrast microscope A 40 × objective was used

The dye test The method introduced by *Sabin & Feldman* (5) was used

Statistics Analyses were performed according to the presentation of the theories of Fisher and others by *Bonnier & Tedin* (5)

The standard deviation was calculated according to the formula $s^2 = \frac{(x-\bar{x})}{n-1}$

EXPERIMENTS

Filtration Experiments

To separate the toxoplasma organisms and the mouse cells of the exudates, different types of filters made of glass fibres, nylon or cotton cloth were used The most satisfactory separation was obtained when filters made of 4 thicknesses of gauze were used Table 1 shows results obtained when an exudate was passed through 5 successive filters Samples were drawn after each filtration and parasites and cells were counted in a haemocytometer

TABLE 1

Results Obtained when Mouse Peritoneal Exudate Was Passed through Gauze Filters

Material	Total number of		Per cent of	
	Parasites $\times 10^{-7}$	Cells $\times 10^{-7}$	Parasites	Cells
Original exudate	8.11	8.34	100	100
Filtrate 1	7.25	5.62	89.4	67.4
2	7.07	5.01	87.2	60.0
3	6.64	4.00	81.9	48.0
4	5.04	3.19	62.1	38.2
5	3.81	2.31	47.0	27.7

When the exudate had passed the second filter, aggregates of parasites were no longer observed After five filtrations two thirds of the mouse cells were removed from the filtrate but concomitantly more than 50 per cent of the parasites were lost

Centrifugation Experiments

Exudates were centrifuged for various periods of time in a refrigerated centrifuge at g values of 65, 260, 440 and 1,030 The number of parasites and cells were counted prior to the centrifugation and then in the sediments and in the supernatants

TABLE 2

Recovery of Parasites and Cells in Sediments and Supernatants after Centrifugation

g values	Time in min	Phase	Per cent recovery	
			Parasites	Cells
65	5	Sediment	22.2	26.9
		Supernate	79.2	68.4
	10	Sediment	29.4	35.1
		Supernate	69.7	65.6
	15	Sediment	40.7	50.0
		Supernate	52.6	47.3
260	10	Sediment	56.1	62.7
		Supernate	50.3	26.9
	15	Sediment	56.5	63.1
		Supernate	39.4	25.6
	20	Sediment	78.4	75.8
		Supernate	16.5	25.7
440	10	Sediment	76.0	77.3
		Supernate	29.9	20.5
	15	Sediment	74.4	78.9
		Supernate	25.6	16.4
	20	Sediment	87.0	79.7
		Supernate	12.9	7.1
1030	10	Sediment	81.1	73.3
		Supernate	11.3	26.7
	15	Sediment	76.6	85.3
		Supernate	8.3	5.5
	20	Sediment	62.5	82.4
		Supernate	7.2	4.9

In Table 2 mean results of two series of centrifugation are presented. As shown by the table the parasites settled at approximately the same rate as the other cellular components. The exudates could, however, be freed from clumps of cellular debris and aggregates of parasites by centrifugation at 65 × g for 5 minutes. After centrifugation at 260 × g for 10 minutes *half the* number of parasites remained in the supernatant while two thirds of the cells were found in the sediment. When spun at 440 × g for 20 minutes approximately 7/8 of the parasites were found in the bottom phase. Centrifugation at 1030 × g resulted in only 70 per cent recovery of parasites. Many of these parasites appeared to be damaged.

The separation of parasites and cells using density gradients met with difficulty. Various gradients on cellulose, sucrose and salt bases were tried, but unsatisfactory loss of parasites was enco[illegible]

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gina [illegible] udate pool. After the centrifugation of the parasite suspensions at 65 × g for 5 minutes the sediment was discarded and the supernatant was spun again at 440 × g for 20 minutes. The supernatant obtained was discarded and the sediment was resuspended

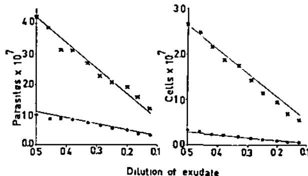


Fig 1

The efficiency of the procedure adopted for the separation of toxoplasma parasites and mouse cells in mouse peritoneal exudates

Various amounts of exudate and tissue culture medium were mixed to a final volume of 6 ml The mixtures were subjected to differential centrifugation (see text) The total number of parasites (left graph) and cells (right graph) were counted before (crosses) centrifugation and after resuspension (circles) The results of the counts were plotted against the reciprocal of the dilution of the exudate The drawn lines represent the values theoretically expected after the dilution of the exudate (upper curve in each graph) and after the centrifugation on the basis of recovery of 30 per cent and 10 per cent of parasites and cells respectively

in medium The suspension was subjected to 260 × g for 10 minutes, the sediment was then discarded, and the supernatant was spun for 20 minutes at 440 × g The sediment obtained was resuspended in medium This suspension should not contain more than 10 per cent of the mouse cells present in the original peritoneal exudate

To see if this was the case various amounts of exudate and medium were mixed, giving final volumes of 6 ml The mixtures were subjected to differential centrifugation according to the schedule described The total number of parasites and mouse cells were counted before and after centrifugation The results are recorded in Fig 1 and show a reasonable good correlation to expected values, represented by the drawn lines

The Effect of the Addition of Serum on the Stability of Toxoplasma

An exudate was divided into 7 portions and each portion was diluted 1 in 2 with one of the 7 following media A 0 5 per cent lactalbumin hydrolysate in Hanks' solution, B medium A with an addition of one per cent dye-test negative human serum, C medium A with an addition of 10 per cent serum, D and E medium A with addtion of 20 per cent serum, F and G medium A with addition of 40 per cent serum The serum added to B, C, D and F was not inactivated while that added to E and G was heated at 56° C for 30 minutes The number of parasites and mouse cells per ml were counted and then the 7 suspensions were subjected to differential centrifugation All 7 suspensions were treated identically After the last centrifugation the parasites and the cells were counted again The results obtained are listed in Table 3

TABLE 3

The Effect on the Recovery of Parasites after Addition of Serum to the Medium

Suspension	Parasites per ml x 10^-6		Cells per ml x 10^-6	
	Original	Final	Original	Final
A	4 58	3 92	3 89	1 01
B	4 68	4 06	3 86	0 93
C	4 90	6 11	3 84	1 10
D	5 16	7 12	3 80	1 20
E	5 14	7 14	3 73	1 26
F	5 12	6 30	3 83	1 05
G	5 04	6 17	3 33	1 21

Parasite containing exudates were diluted with media A G Parasites and cells were counted before (original) and after (final) the suspensions were subjected to differential centrifugation For description of media see text

Addition of serum to the parasite suspensions was found to improve the stability of the parasites markedly This was reflected in the parasite counts performed before as well as after the centrifugations Thus, addition of serum to a final concentration of 10 per cent in the suspensions increased the recovery of parasites after the centrifugation by more than 500 000 per ml Addition of serum to 40 per cent in the medium did not further improve the protective effect The parasites were protected to a similar degree by heated and non heated serum A similar protective effect on the cells was not observed

The Effect on Toxoplasma of Changes in pH

In the experiments described the medium used for the suspending of parasites was adjusted to pH 7 2 by the addition of sodium bicarbonate As the subsequent additions of serum and exudate to the medium were found to influence the pH of the final parasite suspensions the stability of parasites at different pH levels was studied

Different batches of exudates varied in pH between 7 4 and 7 6 and the exudates were found to have a buffering capacity In a series of experiments heat inactivated dye test negative serum was added to some medium which was divided into several portions In each of these the final concentration of serum was 20 per cent The pH was adjusted so that a range from 5 9 to 10 6 was obtained Each of the medium fluids was mixed with exudate in equal proportions This was found to narrow the pH range to 7 2 8 4 Finally the mixtures were subjected simultaneously to identical treatment by differential centrifugation and the parasites were counted

The parasites were found to be most stable at a pH of about 7 9 Lowering the pH to 7 4 or increasing it to 8 4 resulted in a loss of about 40 per cent of the parasites found at the optimal pH (Fig 2)

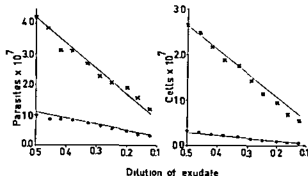


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Practically all parasites could be recovered if a NaCl solution of 0 8 per cent was used With diminishing salt concentration the parasites became gradually more unstable On the other hand the parasites seemed to be fairly resistant to increases of the osmotic pressure Thus, almost 90 per cent of the added parasites were recovered when 2 4 per cent NaCl was used in the mixing fluid at an osmolarity of 610 m osmole

The Effect of Siliconization on the HeLa Cell Cultures

The standardizing effect of silicone treatment was studied by comparing the mean cell number per microscope field in silicone-treated and in untreated cultures At the end of the outgrowth phase and after the renewal of the culture fluid the cells in a number of microscope fields were counted The marginal zones of the cultures were avoided but otherwise the fields were chosen at random In each experiment 5 cultures of each type were observed and 15 fields per culture were studied Thus 15 000 cells in more than 750 fields were counted The cultures were incubated at 37° C and readings were taken 5 times during a period of 24 hours

By the silicone treatment of the slides on which the cells were cultured the area available for cell growth was delimited Cell growth was obtained only on the central area of the slides from which the silicone was removed and the culture fluid constantly covered the cell sheet In Fig 4 the dotted curve indicates the mean number of cells per field using non siliconized Gey chambers The uninterupted curve shows the

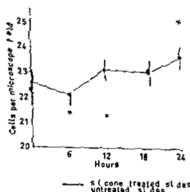


Fig 4

The variation in number of HeLa cells grown on silicone treated and untreated slides

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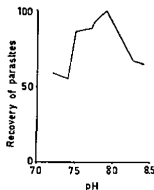


Fig 2

The stability of toxoplasma parasites at different pH levels
Equal portions of mouse peritoneal exudate and tissue culture medium were mixed The pH of the series of mixtures ranged from 7 2 to 8 4 The mixtures were simultaneously centrifuged and the parasites counted in the final suspensions The graph illustrates the stability of parasites at various pH levels relative to that found at pH 7 9 which was plotted as 100 per cent

The Effect on Toxoplasma of Changes in the Osmotic Pressure

A known number of parasites were suspended in tissue culture medium and the pH was adjusted to 7 9 Aliquots of the parasite suspension were mixed with solutions of NaCl or distilled water The series of concentrations of NaCl tested, ranged from 0 2 to 2 4 per cent To 0 1 ml of the parasite suspension 0 2 ml of the particular NaCl solution or distilled water was added and the mixtures were kept at room temperature for 5 minutes The parasites in the different mixtures were then counted and the mean results from three experiments are summarized in Fig 3 The numbers of parasites recovered in the different mixtures are plotted as per cent of the original number against the concentration of NaCl in the salt solutions used

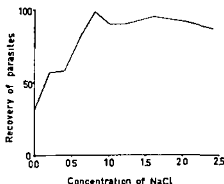


Fig 3

The stability of toxoplasma parasites at different concentrations of NaCl
A known number of parasites suspended in tissue culture medium was added to solutions of NaCl or distilled water The series of concentrations of NaCl tested ranged from 0 2 to 2 4 per cent After 5 minutes at room temperature the parasites were counted and the number of parasites recovered in per cent of those added to the solution was plotted against the concentration of NaCl in per cent

not appreciably different from that in the uninoculated cultures The reduction in cell number in the inoculated cultures, as from the time of inoculation till the reading 19 hours later, was estimated at about three per cent

Twelve batches, each consisting of three cultures, were used to study the variation in cell number The cultures used were inoculated each with about three millions of parasites When the cultures had been incubated for 19 hours the HeLa cells were counted in 10 microscope fields for each culture The results of the cell countings were analyzed statistically

The calculated means of cell numbers per microscope field in each of the 12 batches were found to differ more than could be accounted for by random variation ($P < 0\,001$) On the other hand, the difference between the mean number of cells per field in each of the three cultures within the batches were not statistically significant ($P > 0\,2$) The mean number of cells per field in cultures of the same batch was found to have a standard deviation of 3 9 indicating that when cells in 10 fields of three cultures i e 30 fields are counted for the determination of the mean cell number per field, the standard error ($\frac{s}{\sqrt{n}}$) will be $\pm$ 0 7

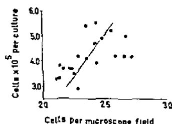


Fig 5

The relationship between the calculated mean cell number per microscope field and the cell number per culture

The mean cell number per microscope field calculated for each of 20 different batches of cultures is plotted against the cell number per culture

The relationship between the mean number of cells per microscope field and the *total cell* number per culture is illustrated by Fig 5 Twenty different batches were studied each consisting of 3 cultures To obtain the figures for the total number of cells in the cultures the cell sheets were disrupted by treatment with

field may be used for an estimation of the total number of cells per culture The mean cell number per culture varied from $2\,9 \times 10^5$ to $5\,5 \times 10^5$

mean number of cells with silicone treated chambers. The vertical lines indicate the confidence limits, $\frac{s}{\sqrt{n}}$. On the untreated slides the mean number of cells varied markedly during the period of observation. In the example given a variation from 21.3 to 25.0 cells per field was found and the standard errors of the mean values varied from 0.50 to 0.70. On the silicone-treated slides the variation of the cell number was less pronounced. In the example given in the figure the mean values varied from 22.1 to 23.6 and their standard errors from 0.40 to 0.49.

Number of HeLa Cells in the Cultures

In a series of 8 experiments the total cell number per culture was estimated. In each experiment 5 silicone-treated cultures were used. The culture medium was replaced by a one per cent trypsin solution, 0.2 ml to each culture. The cultures were incubated for 10 minutes and the fluids were then collected with silicone-treated pipettes. The trypsin treatment was repeated once and the fluids from all of the 5 cultures were pooled. The effectivity of the removal of cells from the cultures was controlled microscopically and the number of cells in the pooled fluids was counted in a haemocytometer. In this manner the cell number was estimated in cultures directly after the outgrowth phase, in cultures which after the outgrowth and renewal of culture medium had been incubated for an additional 19 hours, and in cultures which in addition had been inoculated with 2.5×10^6 to 3.2×10^6 parasites per culture.

TABLE 4

Estimation of the Number of HeLa cells per Culture

Cultures examined	Number hela cells per culture		
	Minimum	Maximum	Mean
After two days of outgrowth	4.51×10^5	4.83×10^5	4.70×10^5
After two days of outgrowth renewal of medium and 19 hours of incubation	4.83×10^5	5.06×10^5	4.94×10^5
After two days of outgrowth renewal of medium inoculation of parasites and 19 hours of incubation	4.20×10^5	4.78×10	4.56×10^5

The results are summarized in Table 4. The figures showing the maximal and the minimal number of observed cells indicate that the variation in results was reasonably small and that the means therefore may be used for purposes of comparison. As shown in the table the number of cells per culture increased insignificantly after the outgrowth phase, and the mean cell number in the inoculated cultures was

the harvest seemed to diminish further the loss of parasites due to coagulation in the exudates

The toxoplasma parasites were found to be most stable in a slightly alkaline medium An optimum was obtained at pH 7 9 *Bimmer et al* (9) studying the variation of dye-test titres with the pH of the added toxoplasma suspension concluded that "if the reaction mixtures are more alkaline the results will be too negative, if they are more acid too positive' One of the reasons for variations in the dye test titres may obviously be the sensitivity of the parasites even to small changes of pH

The multiplication rate of toxoplasma may differ from one line of host cells to another (10, 11) The work of *Shimizu* (7) and our previous results (12) have indicated that variations in the cell number and the age of cell cultures will influence the growth rate and yield of parasites Changes in the tissue culture medium such as variations in the concentration of added serum or renewal of medium in inoculated cultures also seem to affect the growth rate of the parasites

Cell cultures may be used for titration of toxoplasma and for determination of the growth rate of the parasites (13) To evaluate the effect of a certain treatment of the parasites or of the host cells the cell cultures must be standardized satisfactorily and the remaining variations especially in the number of cells must be well known To some extent the standardization of the cultures can be achieved by constancy in the culture conditions such as the use of only one line of a host cell strain, a constant number of cells for seeding, one type of medium and, if serum is used in the medium, the use of only one pool of serum etc

In the present study silicone treatment of the cultures was introduced as a further aid to obtain uniform cell sheets and to reduce the variations in cell numbers between the cultures The results of the cell counts showed that cultures of the same batch are satisfactorily uniform in that respect and consequently the most reliable results will be obtained if cultures from only one batch are used in an experiment The number of cells in cultures of different batches differed significantly Nevertheless a correlation was found between the mean number of cells per microscope field and the total number of cells per culture This indicates that, with [illegible] cell number per f[illegible] per culture Sinc[illegible] presented in a subsequent report (3)—is based on the relation in per cent between the number of parasites that have been able to enter the cells and multiply and the number of host cells available to infection, the correlation mentioned may be useful when results obtained with different batches of cultures are to be compared

DISCUSSION

The mouse exudates contain factors which may exert toxic effects on the cultured cells. Aggregates of parasites and of parasites and other cellular elements are usually present in the exudates. Moreover, white blood cells and cells of the peritoneum present in the exudates often contain parasites at different stages of development. As shown by *Shimizu* (7) the multiplication rate of the parasites is increased if the parasites are washed by centrifugation before the inoculation. To establish conditions permitting reproducibility in studies of the interactions of toxoplasma with its host cell it was, therefore, considered necessary to use suspensions of parasites from which the exudate, the clumps of parasites and the majority of the mouse cells had been removed.

Toxoplasma gondii has a length of 5–6 microns and thus it is not appreciably smaller than many other cellular entities present in the exudates. Ordinary laboratory filters are not satisfactory for separation of parasites and mouse cells. Attempts to use filters made of gauze or nylon cloth for the separation indicated that a higher percentage of the mouse cells than of the parasites adhered to the gauze filters used. Almost as good separating efficiency could, however, be achieved by low speed centrifugation.

By means of differential centrifugation the exudate and the aggregates of parasites could be removed. This was true to a certain extent also for the mouse cells. The parasites seemed to collapse when they were submitted to more than 1,000 × g and the range available for the differentiation of parasites and mouse cells by centrifugation was very small. A procedure could be worked out, however, which allowed the removal of 90 per cent of the mouse cells and yielded parasite suspensions which microscopically appeared to be homogeneous. The 10 per cent of the mouse cells remaining could not be eliminated without excessive loss of parasites.

Toxoplasma is sensitive to lowering of the osmotic pressure below that of a physiological salt concentration. It seems, on the other hand, relatively resistant to an increase of the salt concentration and only a small reduction of the parasite concentration was encountered in the reported experiments when twice the physiological salt concentration was used. However, the parasites were not resistant to the concentrations of salt or sugar which had to be used if separation were to be achieved by means of density gradients.

It has been observed (4, 8) that addition of serum proteins increases the stability of the parasites. In accordance with this observation higher concentrations of parasites were obtained if the exudates immediately after harvest were mixed with a serum containing medium and when suspended in serum containing medium the parasites were more stable during centrifugation. Heparin was added to the exudates to prevent loss of parasites by clogging. The mixing of exudates with medium at

FROM THE MUNICIPAL VIROLOGICAL LABORATORY AND THE VIROLOGICAL LABORATORY OF THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF GOTHENBURG, SWEDEN

A TISSUE CULTURE METHOD FOR TITRATION OF INFECTIVITY AND DETERMINATION OF GROWTH RATE OF TOXOPLASMA GONDII. 2[1]

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ERIK LYCKE and EBBA LUND

Received 21 vi 63

In the present study a method is presented for titration of the infectivity of *Toxoplasma gondii* in HeLa cell cultures Furthermore an exploitation of the method for determination of the growth rate of the parasites is described Methods for standardization of parasite suspensions used as inocula and of the cell cultures have been studied and discussed in a previous report (1)

MATERIAL AND METHODS

The preparation of parasite suspensions The production of mouse peritoneal exudate containing toxoplasma parasites has be n descr...

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[1] This investigation was supported by a PHS research grant AI 05074 01 from the National Institute of Allergy and Infectious Diseases Public Health Service U S A

The skillful technical assistance of Miss Mona Torstensson and Miss Marie-Louise Brodendahl is gratefully acknowledged

SUMMARY

To obtain standardized suspensions of Toxoplasma gondii for use in a tissue culture system, filtration and centrifugation were used to free the parasites from mouse peritoneal exudate The stability during centrifugation, of toxoplasma at various pH levels and at different osmotic pressures was investigated

Attempts were made to standardize the tissue cultures by silicone treatment of the slides on which the cells were grown Finally, the variation of the number of cells in the silicone-treated cultures was estimated

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observed after 7 and 19 hours and the relation of RMIU to dilution of suspension was recorded A linear correlation was demonstrable (Fig 1) between the dilution of the suspension inoculated and the RMIU registered No difference in results was obtained if the cultures were read after 7 hours of inoculation, *i e* when the majority of the parasites had not divided, or if the reading was made after 19 hours and the RMIU was calculated on the basis of counts of solitary parasites as well as clones of parasites In both cases the correlation of RMIU to dilution of the parasite suspension, *i e* the number of parasites inoculated, was good When series of found and expected values of RMIU were compared the difference of the mean values were not statistically significant ($P > 0.5$)

From previously reported studies (1) it is known that, although the batches of HeLa cell cultures were produced in the same way and constant conditions prevailed, a variation was observed in the number of cells between cultures of different batches Between cultures of the same batch this variation was insignificant and seemed to be random A correlation was found between the mean cell number per microscope field and the total cell number per culture (cf Fig 5 in *Lycke & Lund* (1)) This relationship, illustrated in Fig 2, affords the possibility of using the RMIU for calculation of the number of infective parasites in a suspension Examples of this conversion of the RMIU values are given in Table 1

The results obtained with six different suspensions of parasites are compared Six baches of cultures were used, one for each suspension The number of toxoplasma organisms in the suspensions was counted and the cultures were inoculated and incubated After 19 hours of incubation the cultures were read and the RMIU and the mean number

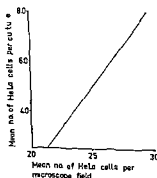


Fig 2

The relation between mean number of HeLa cells per microscope field and mean number of HeLa cells per culture

The figure corresponds to Fig 5 (1) The relationship between cells per culture and cells per microscope field is used for conversion of RMIU to number of infective parasites per inoculum For details see text

To each culture parasites suspended in 0.4 ml medium were added and the cultures were incubated at 37° C.

Readings of the cultures were made after various time intervals. At the readings parasites and cells in 10 different microscope fields of each culture were studied. Phase contrast microscopy using a 40 × objective was employed. The marginal zones of the cultures were avoided at the readings but otherwise the microscope fields were chosen at random.

The number of HeLa cells and the intracellular parasites in each field were registered. When the concentration of infective parasites in a suspension was to be determined the number of parasites that had penetrated the cells was counted. If the reading of the cultures was performed within the first hours after the inoculation, i.e. before any multiplication of parasites had occurred, all the intracellular parasites were counted in each microscope field observed. When a longer interval between the inoculation and the reading had elapsed and multiplication of parasites had begun the solitary parasites—this term is used for toxoplasma organisms which after penetration have not yet divided or are unable to multiply—as well as the clones of parasites were counted. A clone originates from one parasite and when several clones are formed in the cell they appear separated from each other. Each clone was considered an entity in the counts irrespective of the number of constituent parasites in the clone. The numbers of solitary parasites and clones were added for the three cultures and the sum was divided by the number of HeLa cells observed. *Thus a figure for the relation between the number of infective parasites* and the number of exposed cells was obtained. In the following this figure is referred to as the relative number of infective units (RNIU).

When determination of the growth rate of parasites was made the cultures were observed at various times for 19 hours. The number of solitary intracellular parasites, the number of parasite pairs after the first division, and the number of clones containing four or more parasites were registered at each reading.

The number of extracellular parasites was obtained by collecting the fluid phase of the inoculated cultures and counting the parasites in a haemocytometer.

Statistics. The statistical analyses were performed according to the theories of Fisher and others presented by *Bonnier & Tedin* (2).

EXPERIMENTS

The Dose-Response Relationship

Serial dilutions of the parasite suspension were made. As diluent the tissue culture medium was used. Each dilution was inoculated and the cultures were incubated at 37° C for 19 hours. The cultures were

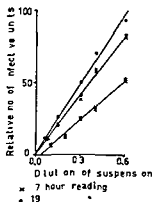


Fig. 1.

The dose response relationship.

The relative number of infective units (RNIU) is plotted against the dilution. Incubation at 37° C.

observed after 7 and 19 hours and the relation of RNIU to dilution of suspension was recorded. A linear correlation was demonstrable (Fig. 1) between the dilution of the suspension inoculated and the RNIU registered. No difference in results was obtained if the cultures were read after 7 hours of inoculation, i.e. when the majority of the parasites had not divided, or if the reading was made after 19 hours and the RNIU was calculated on the basis of counts of solitary parasites as well as clones of parasites. In both cases the correlation of RNIU to dilution of the parasite suspension, i.e. the number of parasites inoculated, was good. When series of found and expected values of RNIU were compared the difference of the mean values were not statistically significant ($P > 0.05$).

From previously reported studies (1) it is known that, although the batches of HeLa cell cultures were produced in the same way and constant conditions prevailed, a variation was observed in the number of cells between cultures of different batches. Between cultures of the same batch this variation was insignificant and seemed to be random. A correlation was found between the mean cell number per microscope field and the total cell number per culture (cf. Fig. 5 in *Lycke & Lund* (1)). This relationship, illustrated in Fig. 2, affords the possibility of using the RNIU for calculation of the number of infective parasites in a suspension. Examples of this conversion of the RNIU values are given in Table 1.

The results obtained with six different suspensions of parasites are compared. Six baches of cultures were used, one for each suspension. The number of toxoplasma organisms in the suspensions was counted and the cultures were inoculated and incubated. After 19 hours of incubation the cultures were read and the RNIU and the mean number

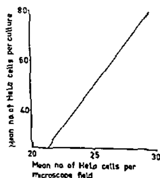


Fig. 2

The relation between mean number of HeLa cells per microscope field and mean number of HeLa cells per culture.

The figure corresponds to Fig. 5 (1). The relationship between cells per culture and cells per microscope field is used for conversion of RNIU to number of infective parasites per inoculum. For details see text.

TABLE 1

The Conversion of the Relative Number of Infective Units (RNIU) to the Number of Infective Parasites

Suspension	Inoculated no of parasites x 10^{-6}	RNIU	Mean cell no per microscope field	Mean no of cells x 10^{-4} per culture	No of infective parasites x 10^{-5}
1	0 88	51 5	27 6	6 7	3 5
2	0 81	51 6	26 4	5 9	3 0
3	1 91	87 8	29 0	7 6	6 7
4	1 33	87 5	26 0	5 6	4 9
5	1 98	90 7	29 4	7 9	7 2
6	1 66	89 7	27 6	6 7	6 0

Six suspensions of parasites were used The concentration of parasites inoculated ranged from 0 81 × 10^6 to 1 98 × 10^6 per culture The mean cell number per microscope field was determined and by means of Fig 2 the mean number of cells per culture exposed to parasitization was calculated This value was used for conversion of the RNIU to number of infective parasites per volume of inoculum

of HeLa cells per microscope field were calculated Knowing the mean cell number per field, the mean number of cells per culture could be obtained from Fig 2 By means of the latter value the RNIU could be converted to the number of infective parasites inoculated

The results listed in Table 1 are chosen in order to show that as a result of the variation in the cell numbers between cultures of different batches, differences in the concentration of parasites between the suspensions may not always be revealed by the RNIU as such Thus, there

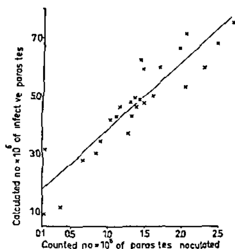


Fig 3

The relation between the concentration of parasites inoculated and the calculated number of infective parasites

The results from the testing of 26 different batches of parasites are plotted in the figure The parasites were counted prior to inoculation The calculation of the number of infective parasites is described in the text

were 1 3 times as many parasites in suspension 3 than in suspension 4, but approximately the same RNIU was recorded for both suspensions When allowance was made, however, for the difference in the number of cells that existed between the two particular batches of cultures, the difference between the calculated numbers of infective parasites amounted to 1 4 as well

In Fig 3 the number of infective parasites in 26 different suspensions was computed in the manner described above and plotted against the concentration of parasites in the suspensions, determined by counting the parasites before the inoculation of the cultures As can be seen a linear correlation was demonstrable The best correlation was obtained if 700,000 to 1 300 000 parasites were inoculated per culture On the average forty per cent of the inoculated parasites could be shown infective with the method used

Multiple Infection

Infection of a host cell with more than one parasite is referred to as a multiple infection If all cells in a culture are equally sensitive to parasitization the occurrence of multiple infections must be dependent on the number of parasites inoculated and on the number of cells available in the culture The multiple infections in an inoculated culture might then be expected to occur according to a Poisson distribution

To see if this was the case the distribution of intracellular parasites in three groups, each consisting of three inoculated cultures was analysed The cultures were incubated for 19 hours at 37° C after the inoculation In each culture 10 microscope fields were observed and the number of uninfected cells was recorded as well as the cells containing one two, three etc solitary parasites or clones of parasites The expected frequency of multiple infection among the latter were calculated according to the formula

$$y = e^{-\bar{x}} \frac{\bar{x}^x}{x!} n$$

in which y denotes the expected number of cells invaded by x parasites when n cells were observed and an average of $\bar{x}$ parasites was found to have penetrated into one cell

Thus six series of data were obtained three showing the found and three the expected occurrence of multiple infected cells The series were compared in χ^2 tests The results of these comparisons, summarized in Table 2 indicated that the differences between found and expected data are probably caused only by chance and thus that all the cells in the culture are equally sensitive to parasitization

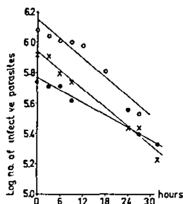


Fig 6

The stability of toxoplasma at 37° C

The log numbers of infective parasites in three different suspensions were plotted against incubation time

In the samples drawn before the suspensions were incubated 0 63 × 10^6 to 1 21 × 10^6 parasites per ml were found infective The inactiva tion of the parasites infectivity, the latter measured by the number of organisms which passed from the fluid of the cultures into the cells was found to proceed at a rate of 2–4 per cent of the parasites per ml and hour (Fig 6)

The results explain the reported inability to account for the parasites inoculated by counting the extra and intracellular parasites They also suggest that the population of parasites is heterogeneous some para sites being more stable than others This difference in stability varied markedly from one batch of suspension to another

Determination of the Multiplication Rate of Toxoplasma

Suspensions of toxoplasma organisms were diluted in tissue culture medium to obtain a concentration of about 2 5 millions of parasites per ml in each suspension Twelve cultures from the same batch were inoculated with each suspension and each culture received about one million parasites The cultures were read after 6 10 14 and 19 hours of incubation At each reading three cultures were observed and then discarded instead of being reincubated Preliminary experiments had shown that inoculated cultures were sensitive to changes in tempera ture

Bursting of cells due to multiplication of parasites was found to occur in cultures incubated for 24 hours but was not observed in cultures in cubated for 19 hours As cell bursts as well as secondary infections would affect the determination of the RMIU the reading of cultures was not performed later than at 19 hours after the inoculation

The RMIU were recorded and grouped according to type of manifesta tion of the infective units As mentioned before the infective units ap peared as solitary parasites or clones containing two or several orga

nisms depending on the time at which the reading was taken and on the multiplication rate of the parasites It seemed therefore possible to study the growth rate by registration of the number of solitary parasites pairs of parasites (the F_1 generation), clones containing 4 parasites (the F generation), clones containing 8 organisms (the F_3 generation) etc In this way a figure was obtained for the number of inoculated parasites able to penetrate the HeLa cells yet undivided or unable to divide Furthermore the numbers of clones containing para sites in the F_1, F_2, F_3 and F_4 generations, respectively, could be used for estimation of the total number of intracellular parasites

In a few instances clones containing more than 16 parasites were noted i e more than 4 divisions had occurred, and they were registered as F_4 generation in order to simplify the calculations

TABLE 3

Example Given to Illustrate Calculations Performed to Estimate the Total Number of Intracellular Parasites the Number of Multiplying Toxoplasma Parasites and the Rate of Multiplication

Time in hours	RNIU	Mean cell no per microscope field	Distribution of RNIU in %					Total no x 10^5 of parasites
			0	F_1	F_2	F_3	F_4	
6	48 4	28 8	99 3	0 7				4 16
10	51 2	28 9	87 4	12 6				4 65
14	54 1	30 6	78 9	14 5	5 8	0 8		5 68
19	52 5	30 1	60 3	22 4	11 2	4 6	1 5	8 68
mean	51 6	29 6						

The table shows the incubation time of inoculated cultures the relative number of infective units (RNIU) recorded and the mean number of cells per microscope field The mean values of the listed RNIU and cells per field were used for con version of RNIU to number of infect
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Table 3 exemplifies the calculations performed The table shows the length of the incubation time of the inoculated cultures, the RNIU re corded and the mean number of HeLa cells per microscope field ob served Previously it was observed that a variation in the cell number between cultures of the same batch was a random phenomenon and that after 6 *hours* incubation of inoculated cultures the additional number of parasites able to penetrate the cells was insignificant This justified the use of mean values of RNIU and cells per field from all the 4 readings to estimate the number of infective toxoplasma orga nisms inoculated In the experiment used to exemplify the calculations the mean values of RNIU and the cells per field were 51 6 and 29 6, respectively According to Fig 2 29 6 cells per microscope field should

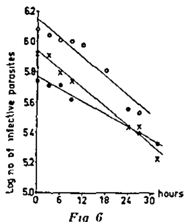


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Bursting of cells due to multiplication of parasites was found to occur in cultures incubated for 24 hours but was not observed in cultures incubated for 19 hours As cell bursts as well as secondary infections would affect the determination of the RNIU, the reading of cultures was not performed later than at 19 hours after the inoculation

The RNIU were recorded and grouped according to type of manifestation of the infective units As mentioned before the infective units appeared as solitary parasites or clones containing two or several orga-

etc) The total number of intracellular parasites was estimated by multiplying 1 13 × 10³ one, two four, eight and sixteen times with the figures listed under 0, F_1, F_2, F_3 and F_4, respectively, and adding the sums The latter are listed on the right hand side of Table 3

Fig 7 summarizes 6 series of results treated as mentioned above Three different suspensions of toxoplasma organisms were used Each suspension was inoculated into two sets of 12 cultures each A different batch of cells was used for each set In the figure the logarithm of the calculated number of intracellular parasites is plotted against time of incubation Figures plotted at time 0 indicate the number of parasites which could enter the host cells

The increase of intracellular toxoplasma organisms proceeded at the same rate irrespective of the subline of HeLa cells used for production of the cultures Small differences were observed if the used suspensions originated from different pools of mouse exudates

The growth rate of toxoplasma cannot be adequately studied, however, from Fig 7 as the results summarized in this figure also include the organisms which after the penetration did not multiply during the period of observation The results used in Fig 7 were, therefore, adjusted by excluding the non dividing parasites and were then replotted in Fig 8 This figure shows that parasites started to multiply after about 6 hours of incubation with a generation time ranging from 6 to 10 hours

DISCUSSION

Cultivation of toxoplasma organisms has so far required the use of living host cells It seems reasonable therefore that the cell culture techniques should be the most sensitive and appropriate methods available *for studies* on toxoplasma growth rate and titration of parasite infectivity *Chaparas & Schlesinger* (3) found that the plaque counting method by *Dulbecco* (4) could be used for quantitative determination of parasite infectivity and that the method was more sensitive and allowed a higher degree of accuracy than other methods hitherto used

Certain difficulties however, are connected with the application of the plaque counting technique to toxoplasma The plaques produced by toxoplasma organisms are small and not clearly visible before 4 to 5 days of incubation (3) Sometimes final titres are not obtained before the twelfth to the fourteenth day (5)

In previous studies (6) it was found that the parasites penetrated the cultured host cells *shortly* after inoculation In the cytoplasm of the host cell the toxoplasma parasite divided [illegible]
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[illegible] observed until 24 hours after inoculation A relationship was discernible between the number of clones observed

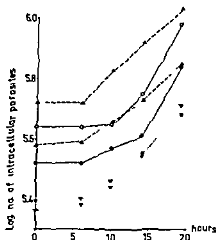


Fig 7.

Estimation of the number of intracellular parasites per culture

Three different suspensions of parasites were each inoculated into two different batches of cultures (filled or unfilled symbols) and incubated at 37° C The log number of intracellularly located organisms was plotted against the incubation time in hours

correspond to 8 0 × 10^5 cells per culture The RNIU was 51 6 and thus 4 13 × 10^5 parasites, *i e.* 39 7 per cent of 1 03 × 10^6 parasites inoculated were found to have penetrated into the host cells

Table 3 also shows the percentage of toxoplasma organisms which had not divided after the penetration and thus were found as solitary parasites (0), and the percentage which had divided and were represented by their offspring of the first, second etc generations (F_1, F_2

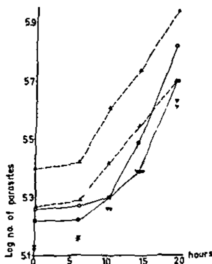


Fig 8

The growth rate of toxoplasma

The results presented in Fig 7 were adjusted by eliminating the number of parasites which did not divide See Fig 7 for explanation of symbols

etc.) The total number of intracellular parasites was estimated by multiplying 4.13×10^5 one, two, four, eight and sixteen times with the figures listed under 0, F_1, F_2, F_3 and F_4, respectively, and adding the sums. The latter are listed on the right hand side of Table 3.

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A relationship was discernible between the number of clones observed

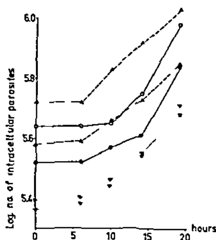


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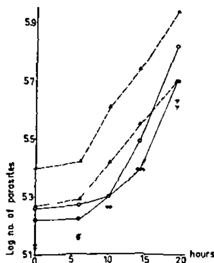


Fig 8

The growth rate of toxoplasma

The results presented in Fig 7 were adjusted by eliminating the number of parasites which did not divide See Fig 7 for explanation of symbols

may be found intracellularly (7). Some of these parasites obviously retain the ability to penetrate the host cells although their capability for multiplication is defective. Also parasites which in the microscope appear to be morphologically intact and in dye tests react as vital organisms have been found devoid of reproductive capacity (8).

The ultimate stage in the degeneration seems to be a disintegration of the parasites. At least this may be inferred from the findings that the number of parasites in a suspension gradually decreases upon storage.

The method described in the present report has been found applicable in studies on the effect of serum factors on toxoplasma (9). In subsequent studies the method will be used for investigation of some of the problems concerning the metabolism of the toxoplasma organism. The method might possibly be used as well in studies on other protozoa requiring a living host cell for reproduction.

SUMMARY

Standardized HeLa cell cultures were used for titrations of standardized suspensions of *Toxoplasma gondii*. A linear dose—response relationship was found and on an average 40 per cent of the inoculated parasites were found infective. The penetration and growth rates of the organisms were studied. The method allowed a distinction between infective and non infective parasites and among the infective parasites between those which multiplied and parasites which did not divide during the period of observation. Observations on the stability to toxoplasma are discussed and possibilities of application of the titration method are mentioned.

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6. *Lund Ebba Lycke F & Sourander P* ... g ndii in cell cultures
7. Lund Ebba *Lycke F & So* ... cultures by means of ... 1961
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in the infected cultures and the concentration of parasites inoculated (5) It was considered worthwhile to investigate if the suspensions of parasites and the cell cultures employed could be sufficiently standardized (1) so that a useful method for titration of infective toxoplasma parasites could be worked out

By the method described in the present report a linear dose—response relationship with a high degree of accuracy was obtained On an average 40 per cent of the inoculated parasites were recovered as infective parasites, i e entered the host cells The plaquing efficiency according to *Chaparas & Schlesinger* (3) varied between 5 and 53 per cent, when inocula from mouse peritoneal exudates were used Using suspensions of parasites produced by artificial lysis of cell cultures for the inoculation, they obtained a predictable efficiency of plating of fifty per cent

With the method reported here results of titrations may be obtained at 6 hours after the inoculation or at any time thereafter since no further penetration of the parasites was observed after six hours Readings could not be performed 24 hours or later after the inoculation, however, as titres obtained then were misleading due to cell bursts and subsequent infection of cells with parasites which had been produced in the culture For practical reasons it was preferable to read the cultures 19 hours after the inoculation

If the development of the toxoplasma infection was followed by repeated observations the cultures used for titration of infectivity could also be used for estimation of the multiplication rate of the parasites When this was done it was found that approximately 50 per cent of the parasites which had penetrated the cells did not multiply during the period of observation Consequently, only about 20 per cent of the parasites inoculated could be shown capable of multiplication Thus, the employed method permitted a distinction within the parasite population not only between infective and non-infective parasites but in addition the infective parasites could be differentiated into organisms capable of multiplication and those which were incapable or at least did not divide during the period of observation The generation time of toxoplasma was found to be in the range of 6 to 10 hours under the experimental conditions applied

As mentioned *Chaparas* and *Schlesinger* could not, even by using artificial liberation of parasites, produce parasite suspensions with more than 50 per cent plaque formers, i e organisms capable of multiplying This is the highest percentage reported and it seems warranted to question whether all parasites generated possess reproductive capacity Outside the host cells the parasites become progressively degenerated Different stages of this process may be distinguished Thus loss of the parasites' motility may be observed within a few minutes after the liberation from the host cell (6) As described in the present study intracellular parasites seem sometimes unable to multiply Similarly the parasites from an irradiated suspension which are unable to divide

may be found intracellularly (7) Some of these parasites obviously retain the ability to penetrate the host cells although their capability for multiplication is defective Also parasites which in the microscope appear to be morphologically intact and in dye tests react as vital organisms have been found devoid of reproductive capacity (8)

The ultimate stage in the degeneration seems to be a disintegration of the parasites At least this may be inferred from the findings that the number of parasites in a suspension gradually decreases upon storage

The method described in the present report has been found applicable in studies on the effect of serum factors on toxoplasma (9) In subsequent studies the method will be used for investigation of some of the problems concerning the metabolism of the toxoplasma organism The method might possibly be used as well in studies on other protozoa requiring a living host cell for reproduction

SUMMARY

Standardized HeLa cell cultures were used for titrations of standardized suspensions of *Toxoplasma gondii* A linear dose—response relationship was found and on an average 40 per cent of the inoculated parasites were found infective The penetration and growth rates of the organisms were studied The method allowed a distinction between infective and non infective parasites and among the infective parasites between those which multiplied and parasites which did not divide during the period of observation Observations on the stability to toxoplasma are discussed and possibilities of application of the titration method are mentioned

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THE TREPONEMATOSES DEPARTMENT, STATENS SERUMINSTITUT, AMAGER BOULEVARD 80, COPENHAGEN S DENMARK

MORPHOLOGY OF THE COLONIES OF T. REITER AND T. KAZAN GROWN IN A SOLID MEDIUM

By

AAGE HEIN CHRISTIANSEN

Received 19 viii 63

T. Reiter and *T. Kazan* are usually cultivated in fluid or semifluid thioglycolate medium containing 10 per cent human or rabbit serum (2). In this medium there are no significant morphological differences between *T. Reiter* and the various strains of *T. Kazan* (II, IV, V, and VIII). *Eagle & Germuth* (4) stated that *T. Reiter* and *T. Kazan* are closely related serologically, but are not identical. *Christiansen* (3) demonstrateed a serological identity between the polysaccharide antigens from *T. Reiter* and *T. Kazan* (strain II) and found an antigen in *T. Kazan* which distinguishes it from *T. Reiter*.

On the basis of these findings, it seemed reasonable to search for other differences between the treponemes in order to contribute to the study of the treponemal taxonomy.

Primarily it was planned to grow the treponemes on the surface of a solid medium in order to se whether possible growth would take the form of colonies in the same way as bacterial colonies. If colonies appeared the intention was to study and compare their morphology with a view to revealing any differences.

Previous Studies

Hoffmann (8) mentioned that in one out of many attempts he noticed a singly colony of spirochaetes on a serum agar plate. In 1921 *Twort* (13) reported one instance of growth of an unnamed spirochaete isolated from a lymph node of a mouse. The medium contained egg and heat-killed *Bacillus phlei* or *Corynebacterium diphtheriae*. The colonies were described as 1–2 mm in diameter with a dull, rough surface and an irregular outline. It is noteworthy that growth took place only under *aerobic* conditions.

In 1923 *Gates* (6) described the cultivation on the surface of blood agar plates of two strains of *Treponema pallidum*, one (ZA) obtained from *Zinsser* (1915) and the other (R) cultivated by *Noguchi* (1911) from the testicle of a syphilitic rabbit. *Gates* noticed two types of colonies, both a minute, raising form and a larger, spreading one. The

raised colonies of the two strains looked similar, while the spreading elements of strain R consistently revealed fimbriae at the edges which were larger than those of strain ZA These treponemes were cultivated in an *anaerobic* jar on the surface of blood agar plates containing 5–7 per cent rabbit blood

In 1928 *Fortner* (5) reported growth of a cultivable strain of *Spirochaeta pallida* on the surface of blood agar using *Bacillus prodigiosus* as oxygen consumer There is no description of the various types of colonies but one picture shows fimbriae at the edge of a colony while the other shows a colony with a regular edge

Fortner's method was used by *Aksjanzew-Malkin* (1), who cultivated the following strains *T Noguchi, T Nichols* (non pathogenic), and *T Aristowsky I and II* The latter strains came from Kazan and one of these is probably now known as *T Kazan II Aksjanzew Malkin* studied the importance of the concentration of the rabbit blood and found 25 per cent to be the optimal concentration The most rapid growth was observed with the Aristowsky strains, after which came the Noguchi and Nichols strains The borderline of the colonies showed a branchlike spreading

Wichelhausen & Wichelhausen (14) only once obtained growth of *T Kazan* on the surface of a blood agar plate, while *T Kazan, T Nichols,* an1 T ...

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on the surface of a

medium containing broth peptone agar, glucose 1 per cent, and 15–20 per cent human serum

Cultivation of treponemes in a solid medium using the stab culture technique was described already in 1912 by *Noguchi* (9, 10) The method was later improved by *Rosebury & Foley* (11)

It has been stated generally that attempts to cultivate treponemes on the surface of a solid medium have met with little success (12)

However, it is difficult to draw a general conclusion from the abovementioned papers concerning the type or species of the strains of treponemes used the morphology of the colonies, and the site of growth It is particularly difficult to decide whether growth has taken place on, in or just below the surface of the plates

MATERIAL AND METHODS

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...n for cultivation in plates is exactly the same except that oxoid ion agar is added to 0 7 per cent (W/V) In this respect attention should be focussed on

THE TREPONEMATOSES DEPARTMENT, STATENS SERUMINSTITUT, AMAGER BOULEVARD 80, COPENHAGEN S DENMARK

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the origin of the serum Horse, ox, rabbit and human sera have been tried The latter two types were excellent, while horse and ox serum could be used only in the seed cultures closed with cotton stoppers which thus contained a certain amount of oxygen Under anaerobic conditions there was no growth at all in a medium containing horse or ox serum

Preparation of Medium

The thioglycolate medium is prepared as described previously (2) Then oxoid ion agar is added up to 0 7 per cent (W/V) and the medium autoclaved After cooling to about 48° C, serum is added up to 10 per cent (V/V) and the medium poured into Petri dishes (diameter 85 mm), with a layer 2–3 mm thick if agar microscopy is to be undertaken Otherwise the layer may be thicker

The anaerobic conditions are created in a metal jar (*Baird & Tallock*, England) closed by a metal cover fitted with a catalyzer After evaporation the jar is filled with a mixture of CO_2 and H_2

One loopful of fluid culture of treponemes is spread on the surface of the plate, which is placed in the jar After establishment of anaerobic conditions the jar is incubated at 37° C Colonies are visible after 2–3 days, and after 7 days they are well developed The growth can be studied microscopically if small pieces of agar are cut out according to the method described by *Ørskov* (16)

RESULTS

General observations Despite careful spreading of the treponemes on the surface of the agar, all the colonies were localized just below the surface and extended down into the agar to varying degrees The same observation concerning the *T Reiter* has been made by *Hardy* (7) A lateral view of a colony revealed an indistinct limitation resembling a root like a piece of cotton Microscopy of the agar showed that the treponemes retired from the colonies by their own movements Formation of secondary colonies in the agar from such liberated treponemes has not been observed during the period of one experiment (7 days) In some of the colonies vacuoles surrounded by treponemes could be observed The colonies of all the strains were white

After 7 days of growth the colonies could be described as follows The colonies of *T Reiter* are ½ 1 mm in diameter and circular (Fig 1) If a vertical section is made through a colony it appears almost to resemble a disc

Microscopy shows a lens-shaped colony The lower convex part is not clear-cut but presents fimbriae surrounded by liberated treponemes

Of the strains studied, *T Reiter* shows the smallest colonies

Fig 2 shows *T Kazan II* The colonies are about four times as large in diameter as those of *T Reiter* The small circle in the centre of the larger colonies is an expression of deeper growth down into the agar, as can be observed by a lateral view of the plate

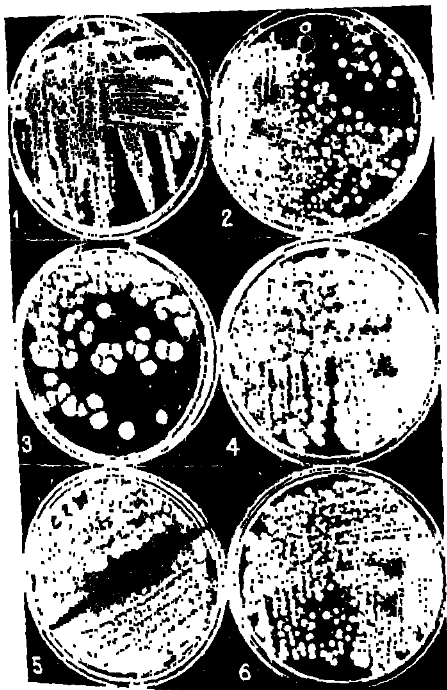

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Fig 3 shows *T Kazan IV* which differs from the above-mentioned strains in having larger and more diffusely spreading colonies

Fig 4 shows *T Kazan V* Two types of colonies are observed, both a larger, diffuse type resembling *T Kazan IV*, and a smaller one resembling *T. Kazan II* After inoculation of new plates from the respective types of colonies, the differentiation into large and small colonies is maintained (Fig 5)

There is apparently no morphological differentiation between the treponemes from these colonies After repeated cultivation of treponemes from the respective colonies the picture remained unchanged Cultivation from large colonies has always resulted in large colonies and vice versa, even for five successive subcultures Therefore, the supposed strain V of *T Kazan* seems to be a mixture of two strains of treponemes

T Kazan VIII (Fig 6) also seems to be a mixed culture of treponemes Repeated subcultivation from the large and small colonies confirmed this impression, and a constant differentiation between large and small colonies was achieved

Finally, the strains of *T Reiter* and *T Kazan* from Professor *Hardy* have been cultivated in the same manner as described above Fig 7 shows a 7-day-old culture of the American strains *T Reiter* (above) looks similar to our laboratory strain, and *T Kazan* resembles our *T Kazan II* judged by the morphology of the colonies

DISCUSSION

It appears to be difficult to compare previous studies with the present experiments Firstly, because very often it is impossible to know for certain which ones of the species of treponemes the various papers are discussing Secondly, it is difficult to be certain whether the treponemes

have been cultivated on or in the agar, and thirdly, growth of the treponemes will depend on the experimental conditions. The previous experiments, which are most similar to the present ones, are those of *Gates* (6), who describes two types of colonies resembling those found with *T. kazan V* and *VIII*. The finding of two types of colonies from these strains raises the question whether this is due to some phase phenomenon, or whether these strains actually are a mixture of two strains of treponemes. Since a change from one type of colony to the other has never been observed in spite of numerous experiments, and since subcultures from the above mentioned small and large colonies always result in the same type of colonies, it is most probable that both *T. kazan V* and *T. kazan VIII* are mixtures of two treponemal strains. It must be borne in mind that the treponemes have been grown at the same time under the same conditions. Minor alterations in the size of the colonies may take place from time to time, but the tendency is always the same, *viz.* the smallest colonies are seen with *T. Reiter* and the larger colonies with *T. kazan*. Three types of colonies may be isolated with certainty from the latter strain (ignoring the above mentioned classification of strains of *T. kazan*). The first type is 2–4 mm in diameter, has a fairly large "fibrous root", and is found with *T. kazan II* and the American *T. kazan*. The second type are the smaller colonies seen with *T. kazan V* and *VIII*. These resemble the first type, except that they are always somewhat smaller. The third type is found in pure culture of *T. kazan IV* and can be recognized as the larger diffuse type of *T. kazan V* and *VIII*. These are the largest colonies of all, being usually from 5 to 8 mm in diameter. It should be stated here that the size of colonies mentioned always refers to single colonies which are expected to have reached full development without suppression from neighbour colonies. The amorphous content of the vacuoles found in some of the colonies is presumed to be some metabolic products of uncertain nature.

Thus the discussion ends with the question whether the original four strains of T. kazan are in reality only three, as may be judged on the basis of the morphology alone. At present this question cannot be answered completely, because a variation in the morphology of the colonies of the strains concerned is not a sufficient basis for classification.

The morphology of the colonies may be of significance in the study of the taxonomy of the treponemes, but the method should be combined with other methods as is well-known from bacteriology. However, the biochemical and physiological methods have not been very well developed as regards treponemes. Thus, attention should be focussed on this main point in order to eliminate the confusion existing concerning the taxonomy of the treponemes.

SUMMARY

A method for the cultivation of *T Reiter* and *T Kazan* strains in a solid medium is reported Differences between the resulting colonies are described, together with three types of colonies found from four strains of *T Kazan*

The method and the findings may be of importance in the study of the taxonomy of the treponemes

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THE NATIONAL INSTITUTE OF HEALTH SEOUL KOREA
AND THE TUBERCULOSIS AND STATISTICAL DEPARTMENTS
STATENS SERUMINSTITUT COPENHAGEN DENMARK

TUBERCULIN PRODUCTION

4 Specificity of Tuberculin of *Mycobacterium tuberculosis* and *M bovis* (BCG Strain)

By

HYUN KYU KIM[1] MOGENS MAGNUSSON and M WEIS BENTZON

Received 2 vii 63

A study has been described previously (*Magnusson, Kim & Bentzon* 1963 b) in which different strains of *M tuberculosis* were cultured on Lind bII medium (*Lind* 1948) The tuberculin activity of the culture filtrates[2] was determined by skin tests on BCG vaccinated guinea pigs, using for each strain the filtrate with the largest protein content from among one to nine week old cultures In the present work, the tuberculin activity of culture filtrate of BCG and three strains of *M tuberculosis* of different geographical origin has been compared by skin tests on four groups of guinea pigs sensitized with each of the four strains, in order to see whether any differences in specificity could be found Purified tuberculin (PPD) was included in the study as standard

For characterization of differences in the specificity of sensitins prepared from strains of *Mycobacterium tuberculosis* and other mycobacteria *Magnusson* (1961) calculated the differences between the homologous and the heterologous reactions to the preparations in guinea pigs sensitized with the strains No difference was found in the specificity of purified tuberculin (PPD) prepared from BCG and from six Danish and four African strains of *Mycobacterium tuberculosis* The same method has been used for characterization of the specificity in the present study

MATERIALS AND METHODS

Strains *C* is a strain of *M tuberculosis* isolated by the Bureau of Animal Industry USA and used extensively in tuberculin production (*Green* 1946) It has been recommended for preparation of tuberculin for veterinary use *Aoyama B* is the only

The authors are great[illegible]
Municipal Hospital of [illegible]

[1] Present address D[illegible]
consin Madison 6 Wisc[illegible]

[2] The term tuberculin activity of culture filtrates is used in the present and some subsequent papers in the same series with essentially the same meaning as the term "tuberculin yield of culture filtrates used previously

SUMMARY

A method for the cultivation of *T Reiter* and *T Kazan* strains in a solid medium is reported Differences between the resulting colonies are described, together with three types of colonies found from four strains of *T Kazan*

The method and the findings may be of importance in the study of the taxonomy of the treponemes

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TABLE 2

Dry Weight of Organism pH and Protein Content of Culture Filtrate of 4 Week Old Cultures of Mycobacterium tuberculosis and M bovis on Lind bII Medium

Organism	Flask no	Dry weight of organism (mg per flask)	Culture filtrate	
			pH	Protein (mg ml)
M tuberculosis E5	1	1700	6 5	0 48
	2	1400	7 1	0 47
M tuberculosis Aoyama B	1	1100	8 4	0 40
	2	1000	8 5	0 40
M tuberculosis C	1	900	9 1	0 29
	2	900	9 1	0 28
M bovis BCG	1	700	8 5	0 34
	2	800	8 6	0 33

The tuberculin activity of the culture filtrates was calculated as in a previous work (*Kim Magnusson & Bent on 1963*) —The amount of tuberculin D_{obs} present in the volume injected is calculated from the formula —

$$\log D_{obs} = \frac{\overline{TR_T} \quad \overline{TR_{ST}}}{b} + \overline{\log D_{ST}}$$

where $\overline{TR_T}$ = average tuberculin reaction for the substance assayed

$\overline{TR_{ST}}$ = average tuberculin reaction for the two standard doses

b = average slope of the tuberculin dose response curve

and $\overline{\log D_{ST}}$ = average of the logarithm of the dose of standard tuberculin (in TU)

The activity D (*in TU per ml of culture filtrate*) is calculated from D = Dilution $\times \frac{1}{\text{volume injected (in ml)}} \times D_{obs}$ using the geometrical mean of the figures calculated for the two doses of culture filtrate used

RESULTS

Bacterial growth and protein content After four weeks strain E5 had reached the largest dry weight of organisms per flask (1400–1700 mg) and BCG the smallest (700 800 mg) (see Table 2) The pH of the filtrate of the BCG strain was 8 5–8 6 9 1 in the cultures of the C strain, and 8 4–8 5, 6 5 7 1 respectively in the filtrates of Aoyama B and E5 The protein content of the filtrates varied from 0 28 mg ml for the C strain to 0 48 mg ml for E5

Tuberculin activity The mean size of reaction after 24 hours was smaller in study A (15 3 mm see Table 3) than in study B (17 2 mm) The doses were the same in the two studies but the volume injected was larger in study B (0 2 ml) than in study A (0 1 ml) In study C the test [illegible] s [illegible] action (16 7 mm Table 3) was [illegible] because the doses were some [illegible] The mean slope varied in the same way as the mean reactions being less in study A than in studies B and C The decrease in mean reaction from 24 to 48 hours was also less in study A than in the two other studies (Table 3)

strain used in Japan for tuberculin production. *E5* was isolated in 1938 from a Danish patient (*Lind* 1947) and is one of the strains used at Statens Seruminstitut for preparation of a series of batches of human purified tuberculin (PPD) including RT 22 and RT 23 (*Lind* 1947, 1948; *Magnusson & Bentzon* 1958). The *BCG* strain was a subculture of the strain used in the BCG Department, Statens Seruminstitut, for preparation of BCG vaccine.

Culture. Two flasks (180 ml of Lind bII medium; *Lind* 1948) of 4 week old cultures of each strain were sterilized by heating in streaming steam for one hour. The
(*Magnus*
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s described

Sensitization of guinea pigs. Four groups each consisting of 20–24 albino guinea pigs bred at the farm attached to Statens Seruminstitut, and weighing 500–600 gm were sensitized with each of the four above mentioned strains according to the method described by *Freund & Gottschall* (1942). Three simultaneous intramuscular injections were given of 0.2 ml of a suspension containing 10 mg of dried heat killed bacteria per ml. The bacteria were suspended in paraffin oil. The injections were given in the neck region and the animals were sensitized 5–6 weeks before being used for intradermal testing.

The animals did not eat for one day after the vaccination. Eleven to twelve days after the vaccination the hind legs of three of the animals sensitized with the C strain were paralyzed and a further nine days later another animal sensitized with the same strain showed similar symptoms. At the same time one guinea pig sensitized with the F5 strain showed a similar defect and after a further 10 days another animal sensitized with the E5 strain was paralyzed in the left front leg. The temperature of the animals was normal. The animals did not recover and three of them were killed three weeks after the vaccination. Histological examination of the brain and spinal cord showed nothing abnormal.[1]

Only healthy animals were selected for skin testing in the present study.

Experimental design. The assay consisted of three studies (see Table 1). In study A the volume injected in each test was 0.1 ml and the culture filtrates were diluted 1:50 and 1:500. In studies B and C the volume injected was 0.2 ml and the dilutions of the culture filtrates 1:100 and 1:1000 and 1:250 and 1:1000 respectively. For each strain equal volumes of culture filtrate from two flasks (see Table 2) were mixed before being assayed. The diluent was phosphate buffered saline pH 7.38 and dilutions of purified tuberculin (PPD) RT 22 Statens Seruminstitut containing 0.00017 mg—10 tuberculin units (TU)—and 0.0017 mg (100 TU: studies A and B) or 0.00067 mg (40 TU: study C) per volume injected were used as standard dilutions.

TABLE 1

Outline of Guinea Pig Studies

Study	Number of animals	Volume injected (ml)	Dilutions of culture filtrates	Standard dilutions
A	20	0.1	1:50 & 1:500	100 TU & 10 TU
B	40	0.2	1:100 & 1:1000	100 TU & 10 TU
C	20	0.2	1:250 & 1:1000	40 TU & 10 TU

In study B the tests with each dilution were distributed over ten test sites on ten animals in each group according to a 10 × 10 Latin square design. In studies A and C the tests with each dilution were distributed at random as regards the site of injection on five animals in each group.

All the injections were given by the same person. The reactions were read after 24 and 48 hours by two readers. The technique used for reading was as described previously (*Kim, Magnusson & Bentzon* 1963). The figures given in this paper are based on the average readings for the two readers.

1 Dr. *Torben Fog*, Chief of the Neurological Department, Municipal Hospital of Copenhagen. Personal communication.

TABLE 5

Mean Si e of Intradermal Reactions Read after 24 and 48 Hours (Frythema in mm) of Guinea Pigs Sensitized with M tuberculosis or BCG and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrates of three Strains of M tuberculosis and BCC

Tubercul n	Number of animals	Number of reactions	Mean reaction* (in mm)		Tuberculin activity of culture filtrates (TU ml)	
			24 hours	48 hours	24 hours	48 hours
E5	80	160	17 2	14 7	90 000	95 000
Aoyama B	80	160	16 9	14 5	75 000	80 000
C	80	160	17 0	14 6	80 000	90 000
BCG	80	160	16 0	13 2	50 000	50 000
PPD RT 22	80	160	15 9	13 0	–	–

* For doses see Table 1

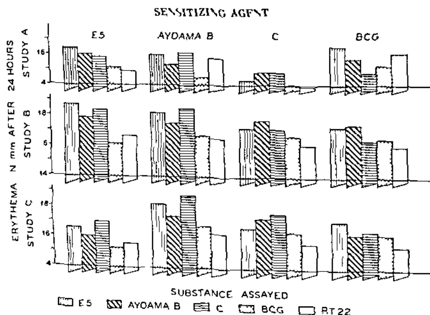


Fig 1

guinea pigs sensitized (BCG strain) to intra details see text)

The mean sizes of the reactions after 24 hours to the standard tuberculin RT 22 viz 15 9 mm and to BCG tuberculin viz 16 0 mm (Table 5) were slightly smaller than to the other three preparations viz 16 9 17 2 mm

The tuberculin activity of the culture filtrates varied from 50 000 TU ml for BCG to 90 000 TU/ml for F5 (Table 5) As already mentioned the protein content of the culture filtrate of BCG was also smaller (Table 2) than that of the other strains The tuberculin activity per mg

TABLE 3

Mean Size of Reactions (Erythema in mm) after 24 and 48 Hours of Guinea Pigs Sensitized with M tuberculosis or BCG to Intradermal Tests with Tuberculin (for further details see Text)

	Number of animals	Number of reactions	24 hours		48 hours	
			Mean reaction (in mm)	Mean slope	Mean reaction (in mm)	Mean slope
Study A	20	200	15 3	3 7	14 6	3 8
Study B	40	400	17 2	5 4	13 9	6 4
Study C	20	200	16 7	5 0	13 6	7 2

Analysis of Variance

Nature of variation	Study A			Study B			Study C		
	d f[1]	Mean square		d f[1]	Mean square		d f[1]	Mean square	
		24hours	48hours		24hours	48 hours		24hours	48hours
Reading error	144	0 42	0 66	288	0 80	0 86	144	0 66	1 21
Residual									
Between animals, same vaccine	144	1 12	1 45	288	2 01	2 17	144	1 79	2 74
Between vaccines	9	2 33*	2 03	9	6 06**	2 72	9	1 98	3 58

1 d f indicates degrees of freedom
* and ** indicate that the variance ratio exceeds the significance level (* 5 per cent, ** 1 per cent)

An analysis of variance has been carried out (Table 3) The variances due to residual errors have been calculated on average values for two readings and therefore comprise one half of the variance due to reading errors, amounting to about 20 per cent The variances are less in study A than in studies B and C The residual variations between animals vaccinated with *different* vaccines were generally larger than between animals vaccinated with the same vaccine This means that the relation between the tuberculins was different for the four vaccine groups However, taking all the studies as a whole, the mean size of reactions in the four groups of animals (Table 4) was very much the same, varying from 16 3 mm in animals sensitized with C strain to 17 0 mm (Aoyama B) after 24 hours

TABLE 4

Mean Size of Reactions (Erythema in mm) after 24 and 48 Hours of Guinea Pigs Sensitized with three Strains of M tuberculosis or with BCG and Subjected to Intradermal Tests with ten Preparations of Tuberculin (see Text)

Animals sensitized with	Number of animals	Number of reactions	Mean reaction (in mm)	
			24 hours	48 hours
F5	20	200	16 7	14 3
Aoyama B	20	200	17 0	14 3
C	20	200	16 3	13 6
BCG	20	200	16 4	13 7

TABLE 5

Mean Si e of Intradermal Reactions Read after 24 and 48 Hours (Erythema in mm) of Guinea Pigs Sensitized with M tuberculosis or BCG and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrates of three Strains of M tuberculosis and BCG

Tuberculin	Number of animals	Number of reactions	Mean reaction* (in mm)		Tuberculin activity of culture filtrates (TU ml)	
			24 hours	48 hours	24 hours	48 hours
E5	80	160	17 2	14 7	90 000	95 000
Aoyama B	80	160	16 9	14 5	75 000	80 000
C	80	160	17 0	14 6	80 000	90 000
BCG	80	160	16 0	13 2	50 000	50 000
PPD RT 22	80	160	15 9	13 0	—	—

* For doses see Table 1

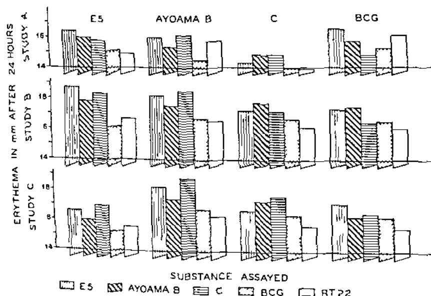


Fig 1

guinea pigs sensitized BCG strain) to intra details see text)

The mean sizes of the reactions after 24 hours to the standard tuberculin RT 22, *viz* 15 9 mm and to BCG tuberculin, *viz* 16 0 mm (Table 5) were slightly smaller than to the other three preparations, *viz* 16 9–17 2 mm

The tuberculin activity of the culture filtrates varied from 50,000 TU ml for BCG to 90 000 TU/ml for E5 (Table 5) As already mentioned, the protein content of the culture filtrate of BCG was also smaller (Table 2) than that of the other strains The tuberculin activity per mg

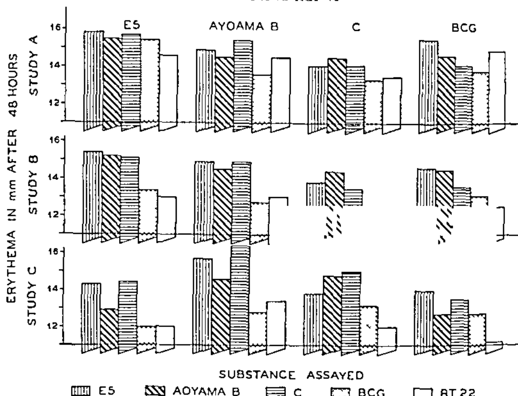


Fig 2

Mean size of reactions (erythema in mm) after 48 hours of guinea pigs sensitized with three different strains of *M tuberculosis* and *M bovis* (BCG strain) to intra dermal tests with culture filtrate of the four strains (for details see text)

TABLE 6

Deviation of Mean Reaction (in mm) to Tuberculin Prepared from four Different Strains from the Total Mean Reaction Obtained in Guinea Pigs Sensitized with the four Strains Average Values at 24 Hours for Studies A B and C (Homologous Reactions are Italicized)

Sensitization of animals	Total* mean (mm)	Tuberculin			
		E5	Aoyama B	C	BCG
E5	16 6	*0 66*	0 00	0 42	—1 09
Aoyama B	17 0	0 43	*—0 24*	0 81	—1 00
C	16 2	—0 14	0 49	*0 18*	—0 53
BCG	16 3	0 63	0 14	—0 44	*—0 33*
Average	16 5	0 40	0 10	0 24	—0 74

* Reactions to PPD RT 22 excluded

of protein was very much the same for all the strains—about 150,000 190,000 TU/mg—except for the C strain, where it was 280,000 TU mg The activities calculated for 24 and 48 hours are similar (Table 5)

The mean sizes of the reactions after 24 and 48 hours with each of the five tuberculins are shown in Figs 1 and 2, for each group of animals

TABLE 7

Specificity Differences of Tuberculin Prepared from three Strains of M tuberculosis and BCG Differences (in mm) between Homologous and Heterologous Reactions Obtained in Guinea Pigs Sensitized with the Strains Used for Preparation of the Tuberculins In Order to Facilitate Reading of the Table all Figures are Shown twice

	Study A				Study B				Study C				Average			
	E5	Aoyama B	C	BCG	E5	Aoyama B	C	BCG	E5	Aoyama B	C	BCG	E5	Aoyama B	C	BCG
24 hours																
E5	–	—0.1	1.2	0.0	–	0.2	0.2	1.9	–	—0.1	0.6	0.5	–	0.0	0.7	0.8
Aoyama B	—0.1	–	—0.8	0.3	0.2	–	—1.6**	—0.1	—0.1	–	—1.1	0.7	0.0	–	—1.2**	0.3
C	1.2	—0.8	–	1.3	0.2	—1.6**	–	0.5	0.6	—1.1	–	1.0	0.7	—1.2**	–	0.9*
BCG	0.0	0.3	1.3		1.9	—0.1	0.5	–	0.5	0.7	1.0	–	0.8	0.3	0.9*	–
48 hours																
E5	–	0.1	0.2	—1.2		—0.3	0.0	0.5	–	0.2	1.1	1.1	–	0.0	0.4	0.1
Aoyama B	0.1	–	—1.3	0.2	—0.3	–	—1.3	0.3	0.2	–	—1.6	1.8	0.0	–	—1.4**	0.8
C	0.2	—1.3	–	0.5	0.0	—1.3	–	0.5	1.1	—1.6	–	1.1	0.4	—1.4**	–	0.7
BCG	—1.2	0.2	0.5	–	0.5	0.3	0.5	–	1.1	1.8	1.1	–	0.1	0.8	0.7	–
Standard errors 24 hrs	0.68				0.64				0.84				0.42			
Standard errors 48 hrs	0.76				0.66				1.04				0.50			

* and ** Indicate that the SPD value deviates from 0 at the 5 per cent and 1 per cent levels respectively

and each study separately. The deviations of the mean reaction for each tuberculin from the total mean (average values at 24 hours for studies A, B and C) are shown in Table 6 for each group of animals. Tuberculin from the C strain gave relatively smaller reactions on BCG-vaccinated animals (deviation: —0.44 mm) and relatively larger (deviation: 0.81 mm) on animals sensitized with Aoyama B strain. The homologous reactions for E5 (0.66 mm) and C (0.18 mm) were slightly larger than the average, whereas those for Aoyama B (—0.24 mm) and BCG (—0.33 mm) were below average. Thus the homologous reactions were not generally larger than the heterologous. This is also evident from Table 7, where the differences between the homologous and heterologous reactions have been calculated for the different pairs of tuberculins. Numerically all the specificity differences (SPD) are small (<2.0 mm). The SPD between Aoyama B and C tuberculin is —0.8, —1.6 and —1.1 mm after 24 hours in the three studies. This is the only case where the SPD deviates significantly from 0 at the 1 per cent level.[1] After 48 hours the SPD values were similar to those after 24 hours (Table 7).

DISCUSSION

No differences in the specificity of tuberculin prepared from different strains of *Mycobacterium tuberculosis* have been found in the present work nor in a series of previous studies, (*Seibert & Morley* 1933, *Jensen & Lind* 1943, *Johnson et al.* 1949, *Asami et al.* 1961, *Magnusson* 1961). It may therefore be sufficient to use a single strain of *M. tuberculosis* for production of tuberculin, as is also the present practice in Japan.

As regards the specificity of human and BCG tuberculin, neither the present study nor some previous studies (*Kurakane & Takasaki* 1954, *Dedebas* 1955, *Magnusson* 1961) revealed any differences. However, other reports (*Lind & Holm* 1943, *Green* 1946, *Magnusson & Lithander* 1949, *Magnusson, Lithander & Hagberg* 1949, *Bluhm* 1949, *Ruziczka* 1952, 1954 a and b, *Quaiser* 1954, *Svenkerud* 1955, *Ohtomo et al.* 1956, *Lorber* 1957, *Lithander* 1958, *Asami et al.* 1961) show that there are small differences in the specificity of BCG tuberculin and human tuberculin, but that these are too small to be of clinical interest. Certain differences in the composition of BCG tuberculin and tuberculin from a virulent strain of *M. tuberculosis* have also been found by electrophoretic analysis (*Tirunarayanan et al.* 1961). It would hardly be justified, therefore, to use the attenuated BCG strain instead of the virulent cultures commonly used for tuberculin production in order to eliminate the risk of laboratory infections, although it might be tempting to do so.

Generally there was good correlation between the results of studies

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[1] The differences between tuberculins varied from study to study in accordance with the variances within groups (see Table 3). Therefore the calculation of standard errors of the specificity differences (Table 7) and the average deviations (Table 6) are based on these variances.

A B and C It was strange that C tuberculin was relatively stronger on Aoyama B animals and weak on C animals (SPD significantly below 0, see pages 247 and 248 However no particular importance can be attached to this observation, since *Asami et al* (1961) did not obtain the same result in a similar study (see Tables 8 and 9) which included some of the same strains as used in the present work

TABLE 8

Deviation of Mean Reaction (in mm) to Tuberculin (OT) Prepared from four Different Strains Obtained in Guinea Pigs Sensitized with the four Strains from the Total Mean Reaction (Homologous Reactions are Italici ed)

Sensitization of animals	Total mean (mm)	Tuberculin OT			
		F 9636	Aoyama B	C	BCG
F9636	14 2	*2 6*	1 1	—1 6	—2 1
Aoyama B	16 1	2 7	*—0 3*	—1 1	—1 5
C	13 2	2 1	0 8	*—0 9*	—2 2
BCG	14 7	3 1	0 3	—2 7	*—0 6*
Average	14 5	2 6	0 5	—1 6	—1 6

Results adapted from *Asami et al* 1961

TABLE 9

Deviation of Mean Reaction (in mm) to Tuberculin (PPD) Prepare l from four Different Strains Obtaine l in Guinea Pigs Sensitized with the four Strains from the Total Mean Reaction (Homologous Reactions are Italicized)

Sensitization of animals	Total mean (mm)	Tuberculin PPD			
		F 9636	Aoyama B	C	BCG
F9636	12 1	*2 2*	—0 3	0 6	—2 6
Aoyama B	12 2	1 5	*1 3*	—0 7	—2 0
C	14 6	2 2	0 2	*1 2*	—3 8
BCG	13 6	1 3	—0 1	—2 6	*1 4*
Average	13 1	1 8	0 3	—0 4	—1 8

Results adapted from *Asami et al* 1961

of

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... previous studies where the tuberculin was concentrated or purified before being examined (*Magnusson* 1961)

When standardizing tuberculin on guinea pigs, a volume of 0 1 ml is usually injected intradermally for each test However, 0 2 ml has also been used (*Green* 1946) It would be expected that the relative variations in the volume injected would be smaller with the larger volume, and volumes of 0 2 ml were therefore injected in the first of the present series of studies (*Kim Magnusson & Bentzon* 1963) However, a number of reactions in that study were not clear and were difficult to read Therefore the results obtained by injecting the tuberculin in volumes

and each study separately The deviations of the mean reaction for each tuberculin from the total mean (average values at 24 hours for studies A, B and C) are shown in Table 6 for each group of animals Tuberculin from the C strain gave relatively smaller reactions on BCG-vaccinated animals (deviation —0 44 mm) and relatively larger (deviation 0 81 mm) on animals sensitized with Aoyama B strain The homologous reactions for E5 (0 66 mm) and C (0 18 mm) were slightly larger than the average, whereas those for Aoyama B (—0 24 mm) and BCG (—0 33 mm) were below average Thus the homologous reactions were not generally larger than the heterologous This is also evident from Table 7, where the differences between the homologous and heterologous reactions have been calculated for the different pairs of tuberculins Numerically all the specificity differences (SPD) are small (<2 0 mm) The SPD between Aoyama B and C tuberculin is —0 8, —1 6 and —1 1 mm after 24 hours in the three studies This is the only case where the SPD deviates significantly from 0 at the 1 per cent level [1] After 48 hours the SPD values were similar to those after 24 hours (Table 7)

DISCUSSION

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showed paralysis of the hind legs and one paralysis of a front leg two to four weeks after sensitization

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of both 0.1 ml (study A) and 0.2 ml (study B) have been compared in the present study. The mean size of reactions, mean slope, standard deviation and reading error were smaller in study A than in studies B and C. That these differences in mean size and mean slope of reaction are actually due to differences in the volume injected has been confirmed in subsequent studies on guinea pigs (unpublished observations).

The volume injected when Mantoux testing humans is usually 0.1 ml. In a study by *World Health Organization/Tuberculosis Research Office* (1957) the volume of diluent of a dose of 5 TU of purified tuberculin (PPD) injected into a human population in South India was varied. By increasing the volume of diluent from 0.05 to 0.2 ml, a slightly clearer separation between large (>12–14 mm) and small reactions (<10 mm) was obtained. *Wijsmuller* (1959) compared the reactions of tuberculosis patients to intradermal tests with a dose of 5 TU of PPD and found larger reactions when the tuberculin was injected in 0.2 ml than in 0.1 ml of diluent.

It would thus appear that in intradermal tuberculin testing the volume of the diluent will influence the mean size of reaction, and possibly the mean slope of the reaction curve and the standard deviation.

No certain explanation of the paralysis of some of the vaccinated guinea pigs, see p. 242, can be given. It is believed that the phenomenon is due to hypersensitivity, and it may probably be seen in relation to experimental allergic encephalomyelitis which can be produced by injecting antigens in Freund's adjuvants containing killed mycobacteria and paraffin oil (*Freund et al.* 1947, 1950). In later studies (*Magnusson* 1962) the vaccine dose has been decreased to 2 or 0.2 mg per animal injected subcutaneously, since when no complications have been seen.

SUMMARY

The tuberculin activity of heat-sterilized culture filtrate of three strains of *Mycobacterium tuberculosis* of different geographical origin and the BCG strain of *M. bovis* on Lind bII medium was compared to that of purified tuberculin (PPD) by intradermal tests on groups of guinea pigs sensitized with the four strains. The relative activity of the culture filtrates varied slightly in the four groups of animals, but the homologous reactions were not systematically larger than the heterologous, and there were hardly any significant differences in the specificity of the four preparations.

The mean reaction, mean slope and standard deviation were larger when a given tuberculin dose was injected in 0.2 ml than in 0.1 ml of diluent.

The animals were sensitized by three simultaneous intramuscular injections of 0.2 ml of a suspension of heat-killed bacteria in paraffin oil, containing 10 mg of dried bacteria per ml. Five out of 90 animals

FROM STATENS SERUMINSTITUT COPENHAGEN STREPTOCOCCAL DEPARTMENT CHIEF KNUD SKADHAUGE M.D AND THE DANISH ATOMIC ENERGY COMMISSION RESEARCH ESTABLISHMENT RISO DENMARK

INACTIVATION OF DRIED BACTERIA AND BACTERIAL SPORES BY MEANS OF IONIZING RADIATION

By

EBBE AHRENSBURG CHRISTENSEN and NIELS W HOLM

Received 2 vii 63

Radiation sterilization is being used increasingly for disposable plastic items and its use has been proposed for food pharmaceutical products surgical sutures etc The field of radiation sterilization has been reviewed in a number of surveys during the last few years (17, 6 36)

As radiation sterilization can be performed at low temperatures it can be applied to products and materials which are damaged by autoclaving and by dry heat sterilization The method can be used in cases of packaged articles and can be performed as a continuous process Radiation sterilization thus seems in many respects to be suitable for sterilization on an industrial basis (17)

INACTIVATION OF MIKROORGANISMS

The biological effect of ionizing radiation depends on the amount of energy absorbed by the irradiated material This energy is measured in rads one rad corresponding to the absorption of 100 ergs per gram of irradiated material (A unit also used frequently is the roentgen (r) corresponding to about 0 93 rad) One Megarad (Mrad) is 10^6 rad

Inactivation of bacteria by irradiation does not always cause the immediate death of the organisms Many biological functions may persist for some hours after the bacteria have been subjected to a dose which prevents their multiplication The generally accepted criterion of sterility however is that growth of microorganisms cannot be demonstrated in liquid or on solid substrates The ability to multiply thus becomes the decisive criterion for inactivation in relation to sterilization

If a few organisms of an originally large population survive irradiation with their ability of multiplicati...

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MATERIAL AND METHODS

The following bacterial strains were used in the experiments
B subtilis Statens Seruminstitut s test strain for routine control of autoclaving dry heat sterilization and formalin sterilization
B globigii ATCC 9372 B subtilis var niger
B pumilus (E 601) made available by Dr *J C Kelsey* Central Public Health

le by Professor A W *Anderson*

de by Professor *R G E Murray*
1961
Corynebacterium selected at random among air borne contaminants in the laboratory

g agar plates
e plates were
ure was then
4 hours The
es to be used
serum broth
(beef broth with 5 per cent ox serum 0 1 per cent glucose and 0 3 per cent horse haemoglobin) The suspensions were adjusted to the desired density and 24 hours after being prepared 0 01 or 0 05 ml of the suspensions were pipetted as drops on
at room temperature in air The dried
contamination by two layers of poly
ach irradiation the uniformity of the
test piece were checked by suspension
at least three untreated and three heat
treated (80° C for 10 minutes) test pieces

Test pieces from the non sporulating strains were prepared by a technique resembling the procedure for preparing spore test pieces as closely as possible
d agar the other strains on Tryptone

ounts of 0 01 ml or n
temperature in air The
tion by double polyethyl ne bags The uniformity and the number of organisms per test piece were controlled by counting the number on three or six test pieces two or three times a week until the experiment was completed The number of bacteria on these controls was always constant during the experimental period

After irradiation the microorganisms were suspended in serum broth using sterile technique and grown on blood agar or TGY agar Conventional dilution and counting technique was used Plates with irradiated bacteria were observed for growth for up to 14 days and broth cultures for up to 30 days (in some experiments up to 60 days)

All irradiations were performed at Risø the Research Establishment of the Danish Atomic Energy Commission either in the Co60plant or the electron accelerator facility The dose in the Co60plant was given with an accuracy of ± 2 per cent and in the electron accelerator facility with an accuracy of ± 10 per cent In the experiments described here irradiation in the electron accelerator facility was only used for the radiation resistant cocci

RESULTS

Inactivation curves were drawn for a total of eight different spore preparations of the same strain of B subtilis Each preparation was examined at various intervals after the preparation was made At the time of their last examination, the oldest spores had been maintained in a dried state for 15 and 12 months, respectively

cumstances, and for the same reason it becomes difficult to compare the results obtained by different laboratories (21, 3).

The environment of the microorganisms before and during irradiation may be of great importance for their resistance The resistance of vegetative forms of bacteria can be increased by anaerobic conditions, drying, high salt concentration, and the presence of proteins during irradiation (10, 5, 4) The radiation resistance of bacterial spores seems in general to be less dependent on the environment than the resistance of vegetative bacteria (17)

With a few exceptions, vegetative bacteria, fungi and their spores are more sensitive to irradiation than bacterial spores Viruses, especially the smaller types, are more resistant than bacterial spores (17, 22) The most radiation resistant microorganism so far described is, however, Micrococcus radiodurans, a pigment producing, non-sporulating tetracoccus This organism was isolated from meat products, it is heat sensitive, *and does not appear to be pathogenic (1, 7, 20, 9) Almost all* the known strains of radiodurans have been found in irradiated canned meat, in which the possibilities of discovering resistant bacteria are good, as individual surviving organisms will be able to multiply and thereby reveal their existence

STERILIZING DOSE

The radiation dose necessary to achieve sterility depends on several factors

1 the species and the numbers of the organisms present before irradiation

2 the environment of the organisms during and after irradiation

3 the standard required, viz the permissible level of surviving organisms

The expression "permissible level of surviving organisms" does not involve a change in the definition of *sterility,* by which is understood, as previously, the absence of living organisms, implying in practice *no demonstrable microorganisms capable of multiplication* The individual object is either sterile or not sterile, but the exponential course of most of the inactivation curves in all methods of sterilization means that it is impossible to indicate any dose whereby all microorganisms will be inactivated All that can be done is to determine the probability of demonstrating microorganisms in a given sample

Based on a study of the effect of autoclaving and on the inactivation curves from irradiation of a considerable number of bacteria, together with the practical experience gained from experiments on sterilization by irradiation, a value of 2 5 Mrad has been chosen in England (17) and USA (2) as a routine sterilizing dose for hospital materials and sutures It is assumed that this dose provides adequate safety under most conditions

MATERIAL AND METHODS

The following bacterial strains were used in the experiments

B subtilis Statens Seruminstitut s test strain for routine control of autoclaving, dry heat sterilization and formalin sterilization

B globigii ATCC 9372 B subtilis var niger

Dr *J C Kelsey* Central Public Health

ailable by Professor *A W Anderson*

... made available by Professor *R G E Murray*, University of Western Ontario November 1961

Corynebacterium selected at random among air borne contaminants in the laboratory

by inoculating agar plates or 48 hours the plates were ours The culture was then mm Hg) for 24 hours The The test pieces to be used the powder in serum broth cose and 0 3 per cent horse haemoglobin) The suspensions were adjusted to the desired density and 24 hours after being prepared 0 01 or 0 05 ml of the suspensions were pipetted as drops on to polyethylene foil and dried for 24 hours at room temperature in air The dried drops were protected against damage and contamination by two layers of poly ethylene foil sealed by welding Before each irradiation the uniformity of the preparations and the number of spores per test piece were checked by suspension dilution and counting of viable units of at least three untreated and three heat treated (80° C for 10 minutes) test pieces

Test pieces from the non sporulating strains were prepared by a technique resembling the procedure for preparing spore test pieces as closely as ...

... dried drops were protected against damage and contamination by double ... er of organisms per six test pieces two number of bacteria

tech ing for ... cultures for up to 30 days (in some experiments up to 60 days)

All irradiations were performed at Risø the Research Establishment of the Danish Atomic Energy Commission either in the Co^{60} plant or the electron accelerator facility The dose in the Co^{60} plant was given with an accuracy of ± 2 per cent and in the electron accelerator facility with an accuracy of ± 10 per cent In the experiments described here irradiation in the electron accelerator facility was only used for the radiation resistant cocci

RESULTS

Inactivation curves were drawn for a total of eight different spore preparations of the same strain of B subtilis Each preparation was examined at various intervals after the preparation was made At the time of their last examination, the oldest spores had been maintained in a dried state for 15 and 12 months, respectively

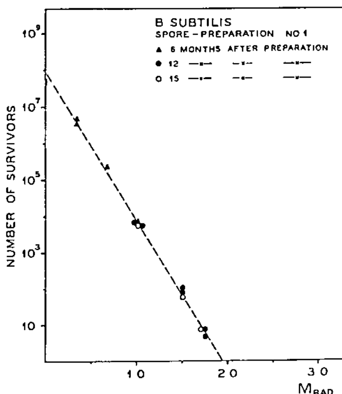


Fig 1
Dried spores of B subtilis (preparation no 1) irradiated in the Co^{60} plant

While the radiation resistance of the individual preparation did not change with time (Figs 1 and 2), the resistance varied from preparation to preparation, even though efforts had been made to maintain uniform conditions of production (Fig 3)

In one experiment, the inactivation curve for B subtilis spores was followed over nine logarithmic steps The curve was linear over its entire course (Fig 4)

The resistance of spores of B globigii and B pumilus was examined in a single preparation of each of these organisms The resistance did not deviate from the mean resistance of the subtilis preparations (Figs 5 and 6)

As was anticipated, the inactivation curves for Staphylococcus aureus and a randomly chosen contaminant (a coryneform rod) were much steeper than the curves for spores (Fig 7) The resistance of various preparations did not vary significantly among themselves, and the slope of the inactivation curves was independent of whether 10^{5} or 10^{8} bacteria were used per test piece

The radiodurans strains were more resistant than the spore preparations examined, and the inactivation curves showed a non linear course (Figs 8 and 9) Different preparations of the same strain gave the same curve form

Using spores of B subtilis, experiments were made to determine whe-

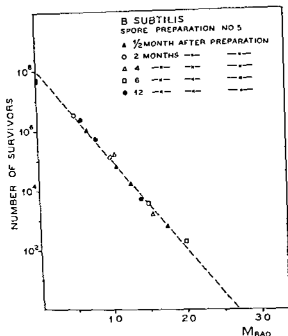


Fig 2

Dried spores of B subtilis (preparation no 5) irradiated in the Co^{60}plant

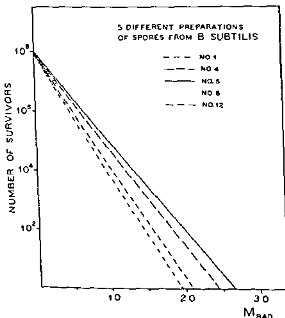


Fig 3

Five different preparations of dry spores from the same strain of B subtilis All irradiated in the Co^{60}plant

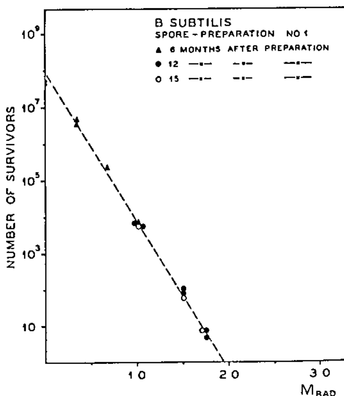


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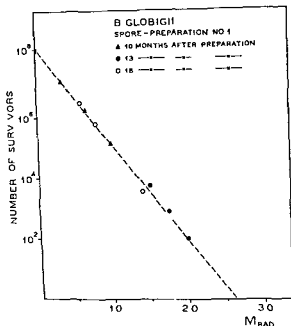


Fig 5
Dried spores of B globigii (ATCC 9372) irradiated in the Co⁶⁰plant

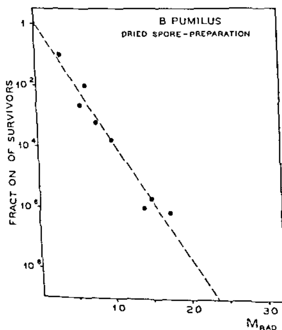


Fig 6
Dried spores of B pumilus (E 601) irradiated in the Co⁶⁰plant

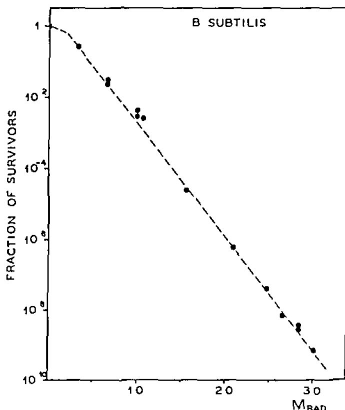


Fig 4

Dried spores of B subtilis irradiated in the Co^{60} plant The experiment was made with 5×10^7 spores per test piece

ther the germination percentage of strongly irradiated spores was the same in serum broth and on blood agar A total of 300 (6×50) test pieces were irradiated in the Co^{60} plant, using six different doses between 2 40 and 3 40 Mrad, so that 50 test pieces all received the same dose One half of the samples were plated on blood agar and the other half were inoculated into serum broth The plates were observed for growth for a period of 14 days and the broth tubes for 60 days The number of positive findings was considerably greater on blood agar than in broth (Table 1) No new colonies appeared on the plates after the 8th day, and after the 17th day no new positive broth tubes was observed

Using spores of B subtilis and B globigii, experiments were made to determine whether the germination percentage on blood agar could be changed by storage under different conditions after the irradiation The tests were made after irradiation with doses between 1 5 and 2 0 Mrad The samples were stored dry Storage for up to six weeks in the dark at 4°, 20° and 37°C had no influence on the number of viable organisms, just as action of the daylight for one, three and five days at room temperature was without influence

In all the strains used in the investigation, irradiated bacteria showed

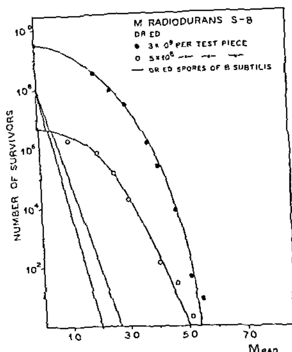


Fig 9

Dried Micrococcus radiodurans (S–8) irradiated in the electron accelerator facility

TABLE 1

Germination of Spores on Blood Agar Plates and in Serum broth after Irradiation by High Doses Fifty Test Pieces with 5 × 10⁷ Spores of B subtilis Were Used at each Dose Level

Dose in Mrad	No of positive tests on blood agar plates (of a total of 25)	No of colonies on blood agar plates total	No of positive tests in serum broth (of a total of 25)
2 46	23	58	11
2 64	10	10	1
2 82	4	4	0
3 00	1	1	0
3 18	0	0	0
3 36	0	0	0

a clear tendency to take longer time to form visible colonies on the plates than non-irradiated bacteria The greater the irradiation dose, the greater was the delay

DISCUSSION

The s\rains examined in the present study showed somewhat greater resistance to ionizing radiation than other workers had found in experiments with strains of the same species (8, 6, 13, 16) This is probably due to differences in experimental technique In the present in

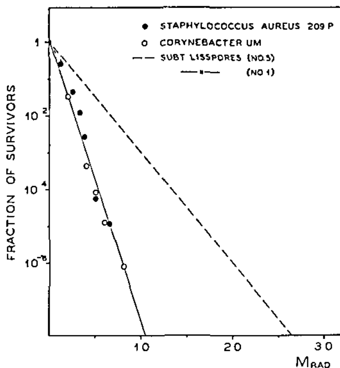


Fig 7

Dried vegetative bacteria Staphylococcus aureus (209 P) and Corynebacterium irradiated in the Co⁶⁰ plant

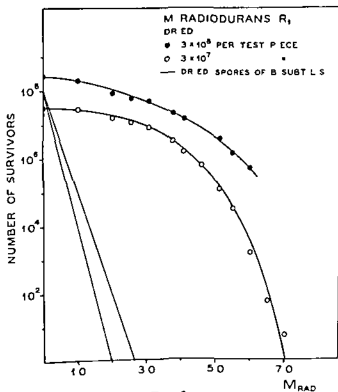


Fig 8

Dried Micrococcus radiodurans (R_1) irradiated in the electr n accelerator facility

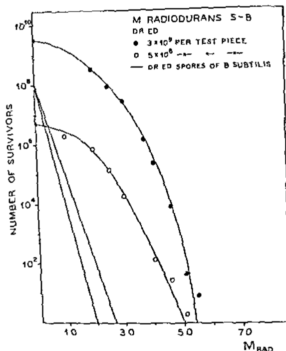


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DISCUSSION

The strains examined in the present study showed somewhat greater resistance to ionizing radiation than other workers had found in experiments with strains of the same species (8, 6 13, 16) This is probably due to differences in experimental technique In the present in-

vestigation, the microorganisms were not washed, and the spores were not heat treated prior to irradiation, as it was considered possible that such procedures might reduce their resistance to irradiation It is well known that bacterial spores exposed to brief heating at a temperature below 100°C germinate more quickly and uniformly than spores which have not been heated Such an activation is commonly used in experiments with spores, also in irradiation experiments, as it kills all vegetative bacteria and makes the determination of spore numbers more rapid and more accurate The activation can however make the spores more sensitive to various bactericidal treatments Cleaning spores by washing and centrifugation can also reduce the resistance to various influences, just as suspension in methanol, which is frequently used in the preparation of "spore paper disks" (11, 12, 16) A preliminary study showed that when subtilis spores were washed in phosphate buffer saline and suspended in methanol before drying, their resistance to ionizing radiation could be reduced by 25 per cent or more It was therefore decided to omit washing and heat treatment Studies on the influence of heat activation and suspension in various liquids on the radiation resistance are in progress

As mentioned, the treatment of the bacteria subsequent to irradiation can be significant for the survival percentage (21, 3) In the present study, the comparison between the growth of irradiated subtilis spores on blood agar and in serum broth was an example of the significance of choice of substrate

The variation in resistance which was observed between various spore preparations of the same subtilis strain might be explained if the strain consisted of a mixture of bacteria varying in resistance, and different substrains had dominated when the preparations had been cultivated Were this the case, it should be possible to isolate substrains of varying resistance This experiment has not yet been attempted Another explanation, however, may be that in spite of efforts to maintain a uniform technique, variations had occurred during the cultivation and the preparation of the spores, for example in the culture medium or in the temperature In general, the heat resistance of spores is correlated with the incubation temperature (14) If spores can alter their radiation resistance as a result of changes in the incubation temperature, this parallels the dependence of heat resistance on temperature during sporulation

As mentioned, in the present experiments the radiation resistance of the individual spore preparations did not alter with time In contrast to this, Proctor et al found that spores of B subtilis (18) and of B thermoacidurans (19) had less resistance to irradiation after being stored A possible explanation of this difference is that Proctor et al stored their spores as stock suspensions in physiological saline at 2–4°C, while in the present investigation the spores were stored dry at room temperature

The strains of radiodurans showed a very high resistance to ionizing radiation in the dry state The number of viable units were only reduced by a factor of 50 or less at 2 5 Mrad The strains isolated so far have been found in canned meat (a total of 78 strains in the course of about three years (1, 7, 20)) and on underwear (one strain (15)) Beyond this nothing is known of the occurrence of such strains, so that their significance in relation to radiation stelilization cannot be evaluated In the above mentioned study of radiation-resistant bacteria in meat, a total of 79 resistant strains were found Of these 78 were cocci and could be classified as belonging to the same species One of the strains however, was a Gram positive, non sporulating rod which seemed to be one of the most resistant of the strains isolated (20)

SUMMARY

The radiation resistance of dried bacteria and bacterial spores has been studied and inactivation curves have been drawn for various strains

The resistance of the subtilis strain examined varied from preparation to preparation, but was constant for more than a year in the individual preparation

In the case of B subtilis spores the fraction of survivors after irradiation was unaffected by variations in storage temperature between 0° and 37°C Illumination (daylight) between 1 and 5 days caused no reactivation of the spores

One of the radiodurans strains examined had an inactivation factor of less than 10 after irradiation with 2 5 Mrad

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FROM VIRUS DEPARTMENT B, NATIONAL INSTITUTE OF PUBLIC HEALTH, OSLO, NORWAY (HEAD A HARBOE, M.D.)

PURIFICATION OF THE NORMAL ALLANTOIC ANTIGEN WHICH REACTS WITH INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

By

ODD KÅRE STRANDLI, KAREN MORTENSSON-EGNUND and ARILD HARBOE

Received 5 vii 63

It has earlier been demonstrated that rabbits injected with normal chick allantoic material, produce antibodies which inhibit the haemagglutination by influenza virus grown in the chick embryo (13, 15) A technique has recently been developed for the titration of the antigen which is present in normal allantoic fluid and reacts with these antibodies, and some of the serological and chemical properties of the host antigen were described (14) This paper presents a method for a partial purification of the host antigen, and gives the results of an analysis of the purified product

MATERIALS AND METHODS

Virus The influenza B strain Lee was used throughout The virus was grown in chick embryos and purified by adsorption to and subsequent elution from fowl red cells

Antiserum Rabbit immune serum was prod[illegible]d by repeated intravenous injec[illegible] strain of influenza A virus *The* [illegible]erae filtrate (Philips Duphar) in[illegible]rbed with 10 per cent fowl red

[illegible]fluenza [illegible]centrifu[illegible]

[illegible] a pre-[illegible] were added in volumes of 0 25 ml, and all dilutions were carried out in a buffered saline made by adding one volume of 0 15 M potassium phosphate buffer pH 7.2 to nine volumes of 0 85 per cent saline

The authors are indebted to Mrs *G Böe* and Mr *A Bye Hansen* for skilled technical assistance

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decrease in the HIN-activity It was possible to extract the antigen at room temperature in a good yield from a dried sample with 10 per cent trichloroacetic acid The crude antigen was, however, precipitated by 50 per cent (w/v) $(NH_4)_2SO_4$ at 4° C, but this did not take place when the antigen had been purified

Resistance of the host antigen to treatment with various enzymes Different enzymes have been used in an attempt to obtain further informations as to the nature of the antigen, β amylase, lysozyme, β glucosidase, β glucuronidase, pectinase, cellulase diastase, hyaluronidase, carboxypeptidase peptidase 'crude' and RNA'se were tried under optimal pH and temperature but in no case was any decrease in activity produced by the action of these enzymes

Purification of the host antigen 4 2 kilogram $(NH_4)_2SO_4$ was added to 7 liter allantoic fluid and stirred overnight at 4°C The precipitate was filtered off by suction and suspended in 140 ml 0 1 M phosphate buffer pH 6 5 containing 0 005 M EDTA and 0 005 M cysteine To this was added 2 g of crude papain (Sigma Chem Corp) in 10 ml of the same buffer The digestion took part overnight at 65°C according to Scott (18)

The suspension was after the digestion heated for ½ hour in a boiling water bath, centrifuged, and the supernatant dialysed overnight against running tap-water at 4°C

The antigen was then precipitated with two volumes of ethanol, and the precipitate dissolved in water The precipitation was repeated twice with subsequent reduction of the water volume which finally was 15 ml All the ethanol precipitations took place overnight at -20°C and in the presence of sufficient amounts of sodium acetate

Subsequently the HIN antigen was precipitated overnight at -20°C

... et al (5) The precipitate was spun down dissolved in water and precipitated with two volumes of ethanol

In order to obtain a further removal of protein, the precipitate was dissolved in an acetate buffer containing 4 per cent (w/v) sodium acetate and 2 per cent (v/v) glacial acetic acid Four volumes of this solution were shaken at room temperature with one volume of a mixture of n butanol and chloroform 1 5 (7) ... no visible layer formed at the in...

The HIN antigen was finally ... nol centrifuged ... for 24 hours agai... dialysed and the yield 50 ... 7 0 ml, ...

Chemical analysis The purified antigen was analysed, and the results are given in Table 1 The HIN titre of the crude allantoic fluid was 200

The HIN titre was defined as the reciprocal value of the antigen dilution which just neutralized the haemagglutination inhibiting activity of the added amount of host specific antibodies

Chemical analysis References to the methods used for the chemical analysis of

N

Identification of non amino sugars The antigen was hydrolysed with 2 N H_2SO_4 in a sealed tube for seven hours in a boiling water bath The hydrolysate was neutralized with solid $BaCO_3$ and centrifuged The supernatant was pipetted off and the sediment washed twice with distilled water The combined supernatant and washings were desalted by passing through a column of Amberlite IR 120 H (The British Drug Houses Ltd) and evaporated to dryness *in vacuo* at 35° C. The residue was dissolved in a small volume of water and applied on Whatman No 1 for paper chromatography The following solvents were used

1 *Pyridin ethyl acetate water acetic acid* (5 5 3 1) according to *Fisher & Dorfel* (8)

2 *Water n butanol acetic acid* (5 4 1)

The chromatograms were developed 15 18 hours in solvent 1 and 72 96 hours in solvent 2 Silver nitrate was used as the spray agent (20)

Identification of the amino sugars Hydrolysis of the antigen was performed with 2 N HCl for six hours in a boiling water bath The hydrolysates were applied on a 0 7 × 35 cm column of Dowex 50 × 12 200–400 mesh (The Dow Chem Comp) and eluted with 0 3 N HCl according to a method by *Gardell* (11) The method separates glucosamine and galactosamine in two peaks

The eluate from each peak was neutralized by adding Zerolitt FF 14 52 mesh in carbonate form (United Water Softeners Ltd) The ion exchanger was spun down in a centrifuge and the neutralized supernatant evaporated to dryness at 35° C *in vacuo* The residue was dissolved in a sufficient amount of water and applied on Whatman No 1 Solvent 1 was used for identification of the hexosamines

Preparation of a Dowex 1 × 8 column Dowex 1 × 8 200 400 mesh (Fluka AG) was used in the chloride form after having been washed several times on a filter with 2 N NaOH 2 N HCl and distilled water in that order A suspension of the washed resin in water was transferred to a column of 1 5 × 17 cm The column was regenerated after use by adding 2 N NaOH distilled water 2 N HCl and distilled water in that order

The column was eluted with NaCl in increasing concentrations Distilled water and 0 25 M NaCl were first employed Gradient elution was then established affording an even increase in chloride concentration from 0 25 to 1 5 M NaCl in the course of 200 ml Chloride concentrations were estimated by titrations with potassium chromate as an indicator Fractions of 5 ml were collected

Preparation of a Sephadex G–200 column Sephadex G 200 columns were prepared according to the directions from the manufacturers (Pharmacia Sweden) Columns of 2 5 × 20 cm were employed and distilled water used for elution Fractions of 5 ml were collected

Electrophoresis This was performed on cellulose acetate strips (5 × 20 cm) in a Shandon electrophoresis apparatus Barbitone acetate buffer pH 8 6 and ionic strength 0 1 was used The current was 4 mA and the electrophoresis was run for 8 hours at room temperature The strips were developed with toluidine blue (17)

RESULTS

Resistance of the host antigen to chemical deproteinization Previous attempts to inactivate the HIN antigen by proteolytic enzymes failed and it was therefore assumed not to be a protein (14) In the present investigation further evidence for this view was obtained by the use of different chemical deproteinization methods Neither extraction with fluorocarbon (12), hot phenol (22) nor a mixture of one part of n butanol and five parts of chloroform (7), did produce any significant

glucosamine HCl, galactosamine HCl, N acetyl glucosamine, N acetyl-galactosamine, N acetyl neuraminic acid, cellobiose, gentiobiose, lactose, maltose, melezitose, melibiose, saccharose, trehalose, raffinose, agar, chondroitin sulphate, heparin and hyaluronic acid were tried None of these revealed any HIN activity

Blood group A saliva has also been tried but did not show any HIN activity neither did the HIN antigen show any blood group A activity

DISCUSSION

It was suggested in a previous paper that the HIN-antigen might be a carbohydrate This conclusion was based on the observation that the antigen was resistant to digestion with proteolytic enzymes and susceptible to oxydation with potassium periodate (14)

The present paper shows that the antigenically active part of the HIN antigen probably is an acid mucopolysaccharide It has, however, not yet been possible to obtain a product which is completely free from protein The protein content has varied from less than two to fifteen per cent in different preparations

The purified product consists of glucose, mannose galactose, fucose, glucuronic acid glucosamine, galactosamine and some sulphate This includes all the monosaccharides which have been reported by other workers to be present in normal allantoic material (1,10) The authors are however, not aware of any earlier report about glucose and glucuronic acid in this material But the unidentified spot which was obtained by Frommhagen et al (10), might have been glucose Glucose has been reported to be present in fowl plague haemagglutinin (24) The absence of sialic acid in the purified product is in agreement with the lack of inhibitory activity

The great number of monosaccharides which have been detected, may indicate that the purified product is a mixture of different acid mucopolysaccharides which may not all be immunologically active So far attempts however have failed to separate these by column chromatography on Dowex 1 × 8 gel filtration on Sephadex G 200 or paper electrophoresis

SUMMARY

A method was developed for the partial purification of the chick allantoic antigen which reacts with the influenza virus HI antibody to host material A chemical analysis of the product was performed, and it was shown that the antigen probably is an acid mucopolysaccharide A small amount of protein was present, but this was assumed to be a contaminant Galactose, glucose, mannose, fucose, glucuronic acid glucosamine, galactosamine, and sulphate were identified

TABLF 1
Analysis of Purified HIN-Antigen

	µg/ml	Units	Method reference	Reference substance
HIN titre		100 000	14	
HI titre		< 20		
Non amino sugars	650 ± 100		23	Galactose mannose (1 1)
Amino sugars	500 ± 150		2	Glucosamine
Hexuronic acid	200 ± 50		3	Glucuronic acid
Methyl pentose	120 ± 25		4	Fucose
Sialic acids	< 20		19	N acetyl neuraminic acid
Protein	75 ± 75		16	Bovine albumin
Sulphate	140 ± 50		6	——
Phosphorous	< 2		9	——

Paper-chromatography of a hydrolysate of the purified antigen The individual monosaccharides were identified by paper-chromatography in solvents 1 and 2 Galactose, glucose, mannose, fucose and glucuronic acid were detected

Due to the desalting with Amberlite IR 120 before applying the hydrolysate on the paper, the hexosamines had been removed These were instead isolated on a Dowex 50 × 12 column according to Gardell (11) Two peaks were found, and the peaks were shown by paper-chromatography to be glucosamine and galactosamine

Column-chromatography on Dowex 1 × 8 Experiments have been performed with column-chromatography on Dowex 1 × 8 in attemps to get a further purification of the antigen No separation was, however, obtained as the HIN-activity was eluted together with the main bulk of material in one, but a very broad fraction extending from 0 6 to 1 2 M NaCl with the main peak from 0 65 to 0 85 M

Gel filtration on Sephadex G-200 This did not give any further purification either The purified preparation which was applied on the column, was eluted in the void volume indicating a high molecular weight on the HIN-antigen

Electrophoresis A single band staining with toluidine blue, was obtained by electrophoresis on cellulose acetate strips in a Shandon electrophoresis apparatus Analysis of the material eluted from this band, revealed no difference in chemical composition to the material applied on the strips

HIN-tests with various known carbohydrates These tests were performed in order to study the structure of the host antigen Solutions of various known carbohydrates were used instead of the host antigen Galactose, glucose, mannose, *fucose glucuronic acid, galacturonic acid,*

FROM THE UNIVERSITY INSTITUTE OF GENERAL PATHOLOGY, COPENHAGEN DENMARK

FUCIDIN®

A Study on Problems of Resistance I

By

K A Jensen and Inger Kier

Received 7 viii 63

In previous works by *Godtfredsen, Jahnsen, Lorck, Roholt & Tybring* (6 7, 8), a comprehensive report is given of Fucidin®, its preparation, as well as a description of its chemical and pharmacological properties and its chemotherapeutic spectrum

At present numerous studies are available on the therapeutical effect of fucidin in cases of mild and severe staphylococcal infections, and even at this early stage, it may be said that this drug is extremely valuable in the therapy of these common infections (4, 10, 11, 12, 13, 15, 16, 17)

Clinical as well as experimental experiences (1, 8, 9, 11, 13, 16), however, have shown that staphylococci can develop resistance to fucidin The object of the present study, therefore, is to elucidate how fucidin-resistant staphylococci emerge, and how to retard the development of this resistance

Even in the first work by *Godtfredsen, Roholt & Tybring* (8) it was demonstrated that when a large inoculum of *Staphylococcus aureus* is added to a concentration of fucidin which is completely bactericidal for a small inoculum, a few cells survive and later multiply The minimum inhibitory concentration of such cultures has been shown to be 64 times or more greater than that of typical cultures *Barber & Waterworth* (1) confirmed these findings, stating "Any large inoculum of staphylococci appears to contain a few mutant cells which showed a considerable degree of resistance"

In analogy with this, *Hilson* (9) reports "There is a relatively high incidence of mutants with varying degrees of resistance to the drugs (a 240 fold increase in one case) Cultures from these grow normally and it is likely that such forms will develop from time to time in patients under treatment with the drug This property suggests that it may be advisable to use fucidin in combination with another suitable drug and treatment should be carried out under strict bacteriological control"

Supported by grants from "*Miss P A Brandt s Foundation*" and *King Christian X s Foundation*

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FROM THE UNIVERSITY INSTITUTE OF GENERAL PATHOLOGY, COPENHAGEN DENMARK

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A Study on Problems of Resistance I

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K. A. JENSEN and INGER KIER

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In previous works by *Godtfredsen, Jahnsen, Lorck, Roholt & Tybring* (6, 7, 8), a comprehensive report is given of Fucidin®, its preparation, as well as a description of its chemical and pharmacological properties and its chemotherapeutic spectrum

At present numerous studies are available on the therapeutical effect of fucidin in cases of mild and severe staphylococcal infections, and even at this early stage, it may be said that this drug is extremely valuable in the therapy of these common infections (4, 10, 11, 12, 13, 15, 16, 17)

Clinical as well as experimental experiences (1, 8, 9, 11, 13, 16), however have shown that staphylococci can develop resistance to fucidin The object of the present study, therefore, is to elucidate how fucidin-resistant staphylococci emerge, and how to retard the development of this resistance

Even in the first work by *Godtfredsen, Roholt & Tybring* (8) it was demonstrated that when a large inoculum of *Staphylococcus aureus* is added to a concentration of fucidin which is completely bactericidal for a small inoculum, a few cells survive and later multiply The minimum inhibitory concentration of such cultures has been shown to be 64 times or more greater than that of typical cultures *Barber & Waterworth* (1) confirmed these findings, stating "Any large inoculum of staphylococci appears to contain a few mutant cells which showed a considerable degree of resistance"

In analogy with this *Wilson* (9) reports 'There is a relatively high incidence of mutants with varying degrees of resistance to the drugs (a 240 fold increase in one case) Cultures from these grow normally and it is likely that such forms will develop from time to time in patients under treatment with the drug This property suggests that it may be advisable to use fucidin in combination with another suitable drug and treatment should be carried out under strict bacteriological control"

Supported by grants from "Miss P A Brandt's Foundation" and "King Christian X's Foundation"

The question is, whether the development of resistance known to occur *in vitro* is parallelled *in vivo* when patients are treated with fucidin exclusively

Clinical reports are available which show that such development of resistance actually takes place

An increase of resistance seems to be particularly common in cases of burns *Lowbury, Cason, Jackson & Miller* (13), treating burns infected by staphylococci, made the following observations In the first series of patients treated twice daily with 500 mg of the drug, staphylococci were eliminated in 15 out of 17 burns in eight patients, but after the treatment, cultures of resistant staphylococci could be obtained from four of the eight patients In the second series 500 mg of fucidin was administered three times daily with a view to preventing any rise in resistance, but after the treatment resistant staphylococci were isolated from at least two out of five patients

√ "Resistant staphylococci of different phage and antibiotic sensitivity pattern were found in different patients, but sensitive and resistant staphylococci from the same burn showed the same or similar patterns From these data it seemed that resistant mutants emerged in more than one of the patients from sensitive precursors during treatment." It is of particular interest that besides finding fucidin-resistant staphylococci in patients treated with fucidin, the occurrence of such resistant staphylococci could be demonstrated in the ward in eight other patients who had not received the drug Hence the author concludes "a policy of controlled use of fucidin combined with another antibiotic is recommended"

After treating about 300 patients (11) suffering from hidrosadenitis furunculosis and—in a few cases—severe staphylococcal infections with fucidin, we were able to isolate fucidin-resistant staphylococci from eight patients from the focal lesion in five cases and from the nose in three We are not able to decide definitely whether the resistant strains found after treatment emerged from the originally sensitive strains, because the phage types have not been determined, but considering the individual patients separately, the strains cultivated before and after treatment were generally found to present the same pattern of resistance except to fucidin

Authors' Experiments

As mentioned above, the first fucidin-resistant strains were found soon after starting the clinical trial, and, consequently, we investigated which other antibiotics might prove suitable in a combination therapy with fucidin *In vitro* experiments using broth with fucidin added in stepwise decreasing concentrations showed, however, fluctuating results In spite of complete inhibition of growth starting at a certain concentration of fucidin, growth was quite often observed in a tube

with a higher concentration of the drug From previous experiments this phenomenon is known to arise from the presence of resistant mutants in the culture

Hence we proceeded to examine the number of resistant staphylococci demonstrable in cultures from patients prior to treatment with fucidin.

TECHNIQUE

A large batch of broth agar was set aside for these experiments, preliminary investigations having shown that the sensitivity of staphylococci may vary from one batch of medium to the other The various antibiotics to be examined were added to the liquefied agar at about 50° C. Each plate was inoculated with about 5 million staphylococci, the number was controlled by inoculation on agar plates without antibiotics The number of colonies was determined after incubation for two and three days at 37° C A period of three days was found most adequate for the reading since colonies of this age showed their characteristic morphology, thus ensuring against contamination by other bacteria Generally the number of colonies would be a little higher after incubation for three days than after two days, but this however was [illegible]

[illegible] originated from patients treated with the drug

RESULTS

The results of the experiments are shown in Fig 1 (see page 278)

From Fig 1 it will be noted that about 1/8 γ of fucidin per ml of medium inhibited growth of almost all the 5 million staphylococci in the inoculum, but that at this concentration and even up to 8 γ per ml a few staphylococci were still able to form colonies In our previous works this has been referred to as "a long tail" These colonies showed a decidedly increased resistance, and the "long tail" is, no doubt, attributable to the presence of resistant mutants in all the 25 examined [illegible]

[illegible] number of resistant colonies, but in a few of the strains *e g* Nos 737 and 1468, the decrease was not marked Furthermore, Fig 1 shows that the concentrations that killed all the 5 million staphylococci varied from 2 to > 8 γ per ml Moreover, no difference was demonstrable between the effects of fucidin on penicillinase-negative and penicillinase-positive strains

The other drugs examined presented quite different features With penicillin —if only penicillinase-negative strains are involved—there is no tailing As regards the penicillinase-positive strains, inhibition is only obtainable at *much* higher concentrations, and even then the borderline of inhibition is not sharply defined Certain [illegible] however [illegible]

TABLE

Penicillinase Negative

	Fucidin				Penicillin				Fucidin 1/2 + penicillin		
	1/2	1/4	1/8	1/16	1/20	1/40	1/80	1/160	1/40	1/80	1/160
P 43	18	45	//	////	0	38	////	////	0	0	0
1673	25	72	//	////	0	200	////	////	0	0	7
737	19	29	54	//	0	200	////	////	0	0	8
1283	31	48	150	////	7	100	///	////	0	0	9
1308	22	30	99	////	0	70	////	////	0	0	0
1358	40	63	109	////	6	100	////	////	0	0	20
1468	7	20	110	////	100	///	////	////	0	0	4
1526	13	17	42	///	19	///	////	////	0	0	3

	Fucidin				Novobiocin				Fucidin 1/2 + novobiocin			
	1/2	1/4	1/8	1/16	1	1/2	1/4	1/8	1/2	1/4	1/8	1/16
P 43	8	28	///	////	0	10	///	////	0	0	0	2
1673	24	47	/	////	0	0	/	////	0	0	1	15
737	19	17	55	///	0	0	/	////	0	0	5	18
1283	18	50	125	////	0	1	//	////	0	0	0	6
1308	12	18	67	////	0	0	/	////	0	0	0	0
1358	16	47	120	////	0	1	///	////	0	0	0	3
1468	12	15	59	////	0	1	0	5	0	0	0	2
1526	13	17	42	///	0	0	0	100	0	0	0	0

	Fucidin				Methicillin				Fucidin 1/2 + methicillin		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1/4
P 43	8	28	///	////	0	17	////	////	0	0	3
1673	24	47	/	////	0	90	////	////	0	0	5
737	19	17	55	///	0	110	////	////	0	1	6
1283	18	50	125	////	0	100	////	////	0	0	5
1308	12	18	67	////	0	34	////	////	0	0	2
1358	16	47	120	////	0	58	////	////	0	2	17
1468	12	15	59	////	0	/	////	////	0	3	4
1526	13	17	42	///	3	200	////	////	0	0	6

	Fucidin				Tetracycline				Fucidin 1/2 + tetracycline		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1/4
P 43	23	65	//	////	0	0	////	///	0	0	0
1625	5	24	103	////	0	8	////	////	0	0	0
1712	7	18	69	////	0	/	////	////	0	0	0
1723	15	44	150	///	0	0	////	////	0	0	0

/ → //// indicate increasing confluent growth
The figures indicate the number of colonies

taphylococci

Fuc 1 n 1 4 + pen 1 n			Fuc 1 n 1 8 + pen c llin			Fuc d n 1 16 + penic llin		
1 40	1 80	1 160	1 40	1 80	1 160	1 40	1 80	1 160
0	0	0	0	0	19	0	100	///
0	0	14	0	0	32	0	100	/
0	0	11	0	5	28	0	34	/
0	0	22	0	0	53	0	//	/ /
0	0	8	0	0	14	0	70	///
0	0	34	0	1	90	100	///	////
0	0	9	0	4	56	30	100	/
0	0	6	0	0	13	0	8	/

Fucl 1 n 1 4 + no ob oc n				Fuc 1 n 1 8 + novob oc n				Fucl 1 n 1 16 + no ob ocin			
1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16
0	0	3	15	0	0	/	////	0	///	////	////
0	0	0	30	0	0	/	////	0	100	/ //	/ //
0	0	2	19	0	0	10	///	0	2	///	////
0	0	0	14	0	0	10	////	0	5	//	////
0	0	0	0	0	0	/	/ //	0	0	//	////
0	0	2	35	0	0	//	////	0	7	/ //	////
0	0	0	0	0	0	0	0	0	0	0	200
0	0	0	0	0	0	0	//	0	0	/	////

Fucid n 1 4 + me hicill n			Fuc 1 n 1 8 + meth c ll n			Fuc d n 1 16 + methic ll n		
1	1 2	1 4	1	1 2	1 4	1	1 2	1 4
0	0	6	0	0	13	0	300	//
0	0	18	0	0	33	0	50	//
0	5	13	0	1	8	0	4	35
0	0	19	0	0	54	0	/ /	///
0	0	7	0	0	21	0	100	///
0	2	19	0	9	110	20	///	/ /
0	3	6	0	31	49	30	//	/ /
0	4	10	0	8	19	0	5	49

Fuci 1 n 1 4 + tetracycl ne			Fucid n 1 8 + tetracycl ne			Fucid n 1 16 + tetracycline		
1	1 2	1 4	1	1 2	1 4	1	1 2	1 4
0	0	10	0	4	69	0	/ /	///
0	0	1	0	8	32	0	/ /	///
0	2	11	0	21	53	0	//	///
0	0	7	0	0	23	0	//	///

TABLE

Penicillinase Negative

	Fucidin				Penicillin				Fucidin 1/2 + penicillin		
	1/2	1/4	1/8	1/16	1/20	1/40	1/80	1/160	1/40	1/80	1/160
P 43	18	45	//	////	0	38	////	////	0	0	0
1673	25	72	//	////	0	200	////	////	0	0	7
737	19	29	54	//	0	200	////	////	0	0	8
1283	31	48	150	////	7	100	///	////	0	0	9
1308	22	30	99	////	0	70	////	////	0	0	0
1358	40	63	109	////	6	100	////	////	0	0	20
1468	7	20	110	////	100	///	////	////	0	0	4
1526	13	17	42	///	19	///	////	////	0	0	3

	Fucidin				Novobiocin				Fucidin 1/2 + novobiocin			
	1/2	1/4	1/8	1/16	1	1/2	1/4	1/8	1/2	1/4	1/8	1/16
P 43	8	28	///	////	0	10	///	////	0	0	0	2
1673	24	47	/	////	0	0	/	////	0	0	1	15
737	19	17	55	///	0	0	/	////	0	0	5	18
1283	18	50	125	////	0	1	//	////	0	0	0	6
1308	12	18	67	////	0	0	/	////	0	0	0	0
1358	16	47	120	////	0	1	///	////	0	0	0	3
1468	12	15	59	////	0	1	0	5	0	0	0	2
1526	13	17	42	///	0	0	0	100	0	0	0	0

	Fucidin				Methicillin				Fucidin 1/2 + methicillin		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1/4
P 43	8	28	///	////	0	17	////	////	0	0	3
1673	24	47	/	////	0	90	////	////	0	0	5
737	19	17	55	///	0	110	////	////	0	1	6
1283	18	50	125	////	0	100	////	////	0	0	5
1308	12	18	67	////	0	34	////	////	0	0	2
1358	16	47	120	////	0	58	////	////	0	2	17
1468	12	15	59	////	0	/	////	////	0	3	4
1526	13	17	42	///	3	200	////	////	0	0	6

	Fucidin				Tetracycline				Fucidin 1/2 + tetracycline		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1/4
P 43	23	65	//	////	0	0	////	////	0	0	0
1625	5	24	103	////	0	8	////	////	0	0	0
1712	7	18	69	////	0	/	////	////	0	0	0
1723	15	44	150	////	0	0	////	////	0	0	0

/ → //// indicate increasing confluent growth
The figures indicate the number of colonies

taphylococci

Fucidin 1 4 + penicillin			Fucidin 1 8 + penicillin			Fucidin 1 16 + penicillin		
1 40	1 80	1 160	1 40	1 80	1 160	1 40	1 80	1 160
0	0	0	0	0	19	0	100	//
0	0	14	0	0	32	0	100	/
0	0	11	0	5	28	0	34	/
0	0	22	0	0	53	0	//	///
0	0	8	0	0	14	0	70	///
0	0	34	0	1	90	100	///	////
0	0	9	0	4	56	30	100	/
0	0	6	0	0	13	0	8	/

Fucidin 1 4 + novobiocin				Fucidin 1 8 + novobiocin				Fucidin 1 16 + novobiocin			
1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16
0	0	3	15	0	0	/	////	0	///	////	////
0	0	0	30	0	0	/	////	0	100	////	///
0	0	2	19	0	0	10	///	0	2	///	///
0	0	0	14	0	0	10	////	0	5	//	////
0	0	0	0	0	0	/	////	0	0	//	////
0	0	2	35	0	0	//	///	0	7	////	////
0	0	0	0	0	0	0	0	0	0	0	200
0	0	0	0	0	0	0	//	0	0	/	////

Fucidin 1 4 + methicillin			Fucidin 1 8 + methicillin			Fucidin 1 16 + methicillin		
1	1 2	1 4	1	1 2	1 4	1	1 2	1 4
0	0	6	0	0	13	0	300	//
0	0	18	0	0	33	0	50	//
0	5	13	0	1	8	0	4	35
0	0	19	0	0	54	0	//	///
0	0	7	0	0	21	0	100	///
0	2	19	0	9	110	20	//	///
0	3	6	0	31	49	30	//	///
0	4	10	0	8	19	0	5	49

Fucidin 1 4 + tetracycline			Fucidin 1 8 + tetracycline			Fucidin 1 16 + tetracycline		
1	1 2	1 4	1	1 2	1 4	1	1 2	1 4
0	0	10	0	4	69	0	///	///
0	0	1	0	8	32	0	//	///
0	2	11	0	21	53	0	//	///
0	0	7	0	0	23	0	//	///

TAB...

Penicillinase Posi...

	Fucidin				Penicillin						Fucidin 1/2 + penicillin					
	1/2	1/4	1/8	1/16	256	128	64	32	16	8	128	64	32	16	8	4
1306	21	37	150	////	7	60	///	////	////	////	0	0	0	1	0	0
1392	4	41	100	////	60	///	////	////	////	////	0	0	0	1	0	0
1552	2	8	21	/	100	////	////	////	////	////	0	0	0	0	0	0
1554	8	22	53	///	38	///	////	////	////	////	0	0	0	0	0	0
1608	11	49	/	////	97	///	///	////	////	////	0	0	0	0	0	0
1759	10	32	120	////	19	112	////	////	////	////	0	0	0	0	0	0
1862	3	10	19	200	///	///	////	////	////	////	0	0	0	0	0	0
2080	2	14	79	////	26	///	////	////	////	////	0	0	0	0	0	0

	Fucidin				Novobiocin				Fucidin 1/2 + novobiocin		
	1/2	1/4	1/8	1/16	1	1/2	1/4	1/8	1/2	1/4	1/8
1306	21	37	150	////	0	2	/	////	0	0	0
1392	4	41	100	////	0	0	//	////	0	0	0
1552	2	8	21	/	0	0	//	////	0	0	0
1554	8	22	53	///	0	0	//	////	0	0	0
1608	11	49	/	////	1	1	/	////	0	0	0
1759	10	32	120	////	1	0	//	////	0	0	0
1862	3	10	19	200	0	1	/	///	0	0	0
2080	2	14	79	////	0	0	//	////	0	0	0

	Fucidin				Methicillin				Fucidin 1/2 + methicillin		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	2	1	1/2
1306	21	37	150	////	0	////	////	////	0	0	0
1392	4	41	100	////	0	////	////	////	0	0	0
1552	2	8	21	/	73	////	////	////	0	0	0
1554	8	22	53	///	11	////	////	////	0	0	0
1608	11	49	/	////	18	////	////	////	0	0	0
1759	10	32	120	////	0	////	////	////	0	0	1
1862	3	10	19	200	300	////	////	////	0	0	1
2080	2	14	79	////	4	////	////	////	0	0	0

	Fucidin				Tetracycline				Fucidin 1/2 + tetracycline		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1...
1306	5	19	77	////	0	0	////	////	0	0	
1392	6	41	105	////	0	/	////	////	0	0	
1554	8	36	78	////	0	3	////	////	0	0	
1608	6	49	200	////	0	/	////	////	0	0	

For explanation of signs see Table 1

2
Staphylococci

Fucidin 1 4 + penicillin							Fucidin 1 8 + penicillin							Fucidin 1 16 + penicillin						
128	64	32	16	8	4	2	128	64	32	16	8	4	2	128	64	32	16	8	4	2
0	0	1	0	1	0	2	0	0	3	5	3	0	21	0	21	//	//	///	///	///
0	0	0	0	0	4	14	0	1	19	17	37	29	67	24	/	//	//	///	///	//
0	0	2	4	2	1	2	0	3	10	2	8	1	9	11	25	35	29	/	55	71
0	0	2	1	0	0	15	0	2	5	7	13	18	53	19	19	[illegible]	34	//	//	//
0	0	0	1	3	21	[illegible]	0	15	16	40	59	58	//	/	//	///	///	////	///	///
0	0	0	0	0	1	1	0	0	3	1	16	4	37	5	/	/	//	//	///	///
0	0	0	0	0	0	1	0	2	4	7	17	3	5	28	41	89	100	/	47	53
0	0	0	0	1	0	1	0	1	1	5	3	0	5	5	12	[illegible]	21	//	///	///

Fucidin 1 4 + novobiocin				Fucidin 1 8 + novobiocin				Fucidin 1 16 + novobiocin			
1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16	1 2	1 4	1 8	1 16
0	0	0	9	0	0	9	///	0	0	/	///
0	0	0	[illegible]	0	0	/	//	0	/	///	///
0	0	0	4	0	0	1	9	0	0	/	//
0	0	0	25	0	0	10	//	0	0	//	////
0	0	0	19	0	0	10	///	0	0	//	///
0	0	0	13	0	0	17	//	0	0	///	////
0	0	0	4	0	0	0	4	0	0	1	/
0	0	0	3	0	0	0	47	0	0	/	//

Fucidin 1 4 + methicillin				Fucidin 1 8 + methicillin				Fucidin 1 16 + methicillin			
2	1	1 2	1 4	2	1	1 2	1 4	2	1	1 2	1 4
0	0	1	13	0	0	8	25	0	0	/	///
0	0	0	11	0	0	3	15	0	0	35	///
0	0	1	4	0	0	3	4	0	0	13	37
0	0	4	6	0	0	6	24	0	0	26	///
0	0	6	18	0	0	4	53	0	35	///	///
0	0	9	9	0	0	21	35	0	19	////	///
0	0	3	5	0	0	5	8	0	1	18	41
0	0	0	1	0	0	3	14	0	0	23	19

Fucidin 1 4 + tetracycline			Fucidin 1 8 tetracycline			Fucidin 1 16 + tetracycline		
1	1 2	1 4	1	1 2	1 4	1	1 2	1 4
0	0	0	0	0	3	0	//	//
0	0	7	0	3	52	0	/	/
0	0	8	0	8	100	0	/	/
0	1	6	0	13	150	0	//	///

TABLE

Penicillinase Positive

	Fucidin				Penicillin						Fucidin 1/2 + penicillin						
	1/2	1/4	1/8	1/16	256	128	64	32	16	8	128	64	32	16	8	4	2
1306	21	37	150	////	7	60	///	////	////	////	0	0	0	1	0	0	0
1392	4	41	100	////	60	///	////	////	////	////	0	0	0	1	0	0	0
1552	2	8	21	/	100	////	////	////	////	////	0	0	0	0	0	0	0
1554	8	22	53	///	38	///	////	////	////	////	0	0	0	0	0	0	3
1608	11	49	/	////	97	///	///	////	////	////	0	0	0	0	0	0	8
1759	10	32	120	////	19	112	////	////	////	////	0	0	0	0	0	0	0
1862	3	10	19	200	///	///	////	////	////	////	0	0	0	0	0	0	0
2080	2	14	79	////	26	///	////	////	////	////	0	0	0	0	0	0	0

	Fucidin				Novobiocin				Fucidin 1/2 + novobiocin			
	1/2	1/4	1/8	1/16	1	1/2	1/4	1/8	1/2	1/4	1/8	1/16
1306	21	37	150	////	0	2	/	////	0	0	0	0
1392	4	41	100	////	0	0	//	////	0	0	0	4
1552	2	8	21	/	0	0	//	////	0	0	0	1
1554	8	22	53	///	0	0	//	////	0	0	0	7
1608	11	49	/	////	1	1	/	////	0	0	0	3
1759	10	32	120	////	1	0	//	////	0	0	0	1
1862	3	10	19	200	0	1	/	///	0	0	0	2
2080	2	14	79	////	0	0	//	////	0	0	0	1

	Fucidin				Methicillin				Fucidin 1/2 + methicillin			
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	2	1	1/2	1/4
1306	21	37	150	////	0	////	////	////	0	0	0	5
1392	4	41	100	////	0	////	////	////	0	0	0	0
1552	2	8	21	/	73	////	////	////	0	0	0	0
1554	8	22	53	///	11	////	////	////	0	0	0	2
1608	11	49	/	////	18	////	////	////	0	0	0	1
1759	10	32	120	////	0	////	////	////	0	0	1	0
1862	3	10	19	200	300	////	////	////	0	0	1	1
2080	2	14	79	////	4	////	////	////	0	0	0	0

	Fucidin				Tetracycline				Fucidin 1/2 + tetracycline		
	1/2	1/4	1/8	1/16	2	1	1/2	1/4	1	1/2	1/4
1306	5	19	77	////	0	0	////	////	0	0	0
1392	6	41	105	////	0	/	////	////	0	0	0
1554	8	36	78	////	0	3	////	////	0	0	0
1608	6	49	200	////	0	/	////	////	0	0	0

For explanation of signs see Table 1

With regard to the penicillinase resistant drug methicillin, there is no tailing worth mention—independently of the penicillinase production

Tetracycline (Terramycin®) and novobiocin show no tailing, and they have the same effect on penicillinase-negative as well as on penicillinase-positive strains

Later we shall revert to the question, whether the *in vitro* presence of 'a long tail' is tantamount to a rapid development of resistance to the drug in patients

The following experiments were carried out to investigate whether the fucidin-resistant mutants are killed if fucidin is combined with another drug Broth agar plates to which various concentrations of fucidin, penicillin, methicillin, novobiocin, and tetracycline were added, either alone or in combination were inoculated with about 5 million staphylococci, and the number of colonies was determined after incubation for two to three days at 37° C The concentrations are shown in Table 1, comprising the penicillinase negative strains, and in Table 2, comprising the penicillinase-positive ones

From Table 1 it will be seen that concentrations of penicillin, methicillin, novobiocin, and tetracycline—which alone caused no appreciable inhibition—killed the fucidin resistant mutants when combined with fucidin The combination of fucidin + penicillin gives the most satisfactory effect Moreover, Table 1 shows that the higher the fucidin concentration employed, the lower the concentration that is required of the other drugs to kill th
apparent that on plates wit
confluent growth—the com
other drugs gives strong inhibition Thus the combination both kills the fucidin-resistant mutants and has a synergistic effect

The conclusions drawn from combining fucidin with novobiocin, methicillin, and tetracycline also apply to findings recorded in Table 2, comprising the penicillinase-positive strains

When penicillin forms part of the combination, the dose required to kill the fucidin resistant mutants is four units, and then only when the highest dose, 0 5 γ, of fucidin is used

DISCUSSION

Our preliminary experiments have shown that quite a number of fucidin resistant mutants were present in all the 25 staphylococcus strains examined Hence, great importance should be attached to the problem, whether an antibiotic, *e g* fucidin, which *in vitro* presents "a long tail , also in human organisms infected with staphylococci selectively can provoke the development of resistance to the agent—in contrast to drugs not presenting such "a long tail"

It is beyond the scope of the present study to provide a definite solut-

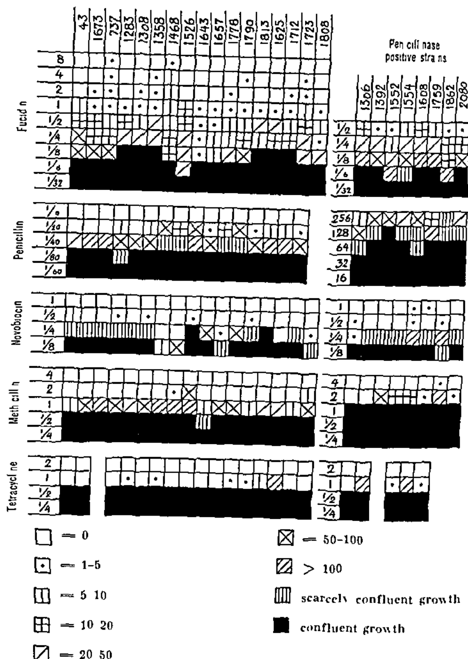


Fig 1

The Concentration of Fucidin Novobiocin Methicillin and Tetracycline in µg per ml and of Penicillin Units per ml of Medium

is dependent upon the size of the inoculum, and as it is difficult to spread the inoculum uniformly on the surface of the medium groups of small colonies developed on some plates that otherwise showed complete inhibition However, this did not cause any trouble in the study of the combined effect of fucidin and penicillin

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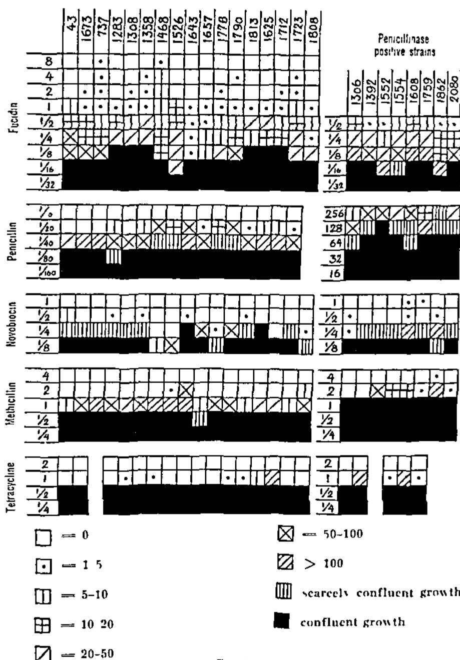


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From our previous studies it is also apparent that INH- or streptomycin resistant tubercle bacilli readily develop during treatment of cavernous tuberculosis, when only one of the drugs is used These investigations showed that resistance developed within three months in almost all of the patients treated

From Fig 1, however, it will be seen that tetracycline gives a sharp borderline of inhibition, although it is evident from Table 3 that 15 3 per cent tetracycline-resistant staphylococci were cultivated from patients treated in the out-patients' clinic Inoculation of only 5 million bacteria does not, however, exclude the occurrence of resistant mutants In further experiments with tetracycline using a larger inoculum than 5 million bacteria, a few tetracycline-resistant mutants were occasionally found

Thus the primary question may be answered as follows it can hardly be doubted that bacterial cultures containing numerous mutants resistant to a certain antibiotic are more prone by far to development of resistance both *in vitro* and *in vivo*, if exposed to the drug concerned

The greatest caution should be exercised when such drugs are used and it is important that future tests for "tailing" be carried out whenever new drugs are introduced

The incidence of these resistant mutants makes it relevant, however, to ask why resistance is not developed in all staphylococcal infections treated with fucidin alone? In order to explain this phenomenon all the factors involved in the development of resistance should be discussed

One of these factors is the amount of staphylococci present in the human body in the various types of staphylococcal infections

From Tables 1 and 2 it is seen that the number of fucidin resistant mutants per 5 million staphylococci falls between a few to about one hundred Five million staphylococci occur readily in staphylococcal infections, even in cases of furunculosis In more severe staphylococcal infections, *e g*, sepsis, pneumonia, or large abscesses, the bacterial populations concerned will be so great in each case that numerous resistant mutants will be present It is a fact, however, that resistant staphylococci develop only in some of the patients treated with fucidin alone, and hence a factor must be involved which operates in the opposite direction This factor is the natural and acquired resistance of the organism

Upon treatment with fucidin, all the sensitive staphylococci are killed and thus the number of staphylococci in the organism is greatly reduced In this way, the organism reassumes its normal, and later its acquired, resistance in order to cope with the remaining resistant mutants Conversely, if a staphylococcal infection develops in the preformed cavities of the organism or in large abscesses, the staphylococci will be located outside the reactive tissue, where the resistant mutants can multiply An example of this is seen in the high incidence of fucidin-resistant mutants in burns, where the staphylococci

ion to the problem, but the following investigations and arguments seem to indicate that "a long tail" predisposes to development of resistance

Fucidin presents "a long tail" and, as mentioned in the introduction, fucidin-resistant staphylococci have been found to develop rapidly and rather frequently after treatment, particularly of burns, and even the beginning of a hospital epidemic caused by resistant staphylococci has been recorded Our own therapeutical experiences did not present the same incidence but, considering the very brief period in which fucidin has been used combined with the fact that our patients were treated in an out-patients' clinic, the incidence is sufficient to justify the assertion that the easily practicable *in vitro* test discussed in the present work has directed our attention to the possibility of such occurrences We did not obtain this information from determinations of resistance by means of the disc method on about 1000 strains of staphylococci isolated from patients prior to treatment with fucidin On the contrary, the sensitivity to fucidin of all these strains was equal, or superior, to the sensitivity of our standard strain Occasionally, however, a few colonies may be seen within the zone of inhibition, and at subsequent resistance determinations these colonies contained staphylococci more resistant to fucidin than the rest of the culture

TABLE 3
The Resistance of Staphylococci Cultivated before Treatment from the Same Number of Patients

	Per cent resistant to				
	Streptomycin	Penicillin	Tetra cycline	Chloram phenicol	Fucidin
Focus 406 strains	40 9	65 5	15 3	2 7	0
Nose 273 strains	36 6	69 2	16 5	4 4	0

Like fucidin, streptomycin was found to have "a long tail", whereas tetracycline presented no tail Hence in Table 3 we have recorded the resistance of all staphylococcal strains isolated from patients during their initial examination in the out-patients clinic This table shows that 40 9 per cent of the staphylococcus strains were resistant to streptomycin and 15 3 per cent to tetracycline, even though streptomycin generally is not employed as often as tetracycline

The following example illustrates the readiness with which staphylococci develop resistance to streptomycin An outbreak of a widespread staphylococcal epidemic occurred in a tuberculosis hospital All the staphylococci cultivated from these cases were found to be resistant to streptomycin

In experiments that will be published later, INH and streptomycin showed a rather "long tail" against tubercle bacilli

From our previous studies it is also apparent that INH or streptomycin resistant tubercle bacilli readily develop during treatment of cavernous tuberculosis, when only one of the drugs is used These investigations showed that resistance developed within three months in almost all of the patients treated

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are probably localized outside the reactive tissue Another example is the *development of streptomycin- and INH-resistant tubercle bacilli* during the treatment of cavernous pulmonary tuberculosis on employment of streptomycin or INH alone Hence it can hardly be doubted that the resistance of the organism is the main reason why resistant staphylococci do not develop in all fucidin-treated cases

In the following the significance of the development of resistance for the treatment of different kinds of infections is discussed

Two factors are important for the rate at which chemo-resistant bacteria spread in a human population, 1) the rate of the infection and 2) the virulence Firstly, we know that the staphylococci spread rapidly, and that the rate of infection is high Secondly, a selection of resistant mutants takes place during treatment Simultaneously, a selection of the most virulent staphylococci takes place, simply because they are able to invade the human organism and cause disease Primarily this will start in hospitals, but later spread to the population

The wide significance of these two factors has already been apparent in the severe hospital infections attributable to chemo-resistant and virulent staphylococci

In order to prevent a selection of chemo-resistant and virulent staphylococci, it is of the utmost importance to find one or more other antibiotics which may be used in combined therapy with a given antibiotic

The present study shows that fucidin and penicillin are suitable for combined treatment of infections caused by penicillinase-negative staphylococci

But the high percentage of existing penicillinase-producing staphylococcus strains (cf Table 3) that are not recognizable until resistance determination has been carried out, makes it advisable in cases of severe and rapidly progressing staphylococcal infections to combine fucidin with a penicillinase-resistant penicillin, *e g*, methicillin Parenteral administration of high doses of penicillin may perhaps have the same effect

The comprehensive experience obtained in Blegdamshospitalet (Copenhagen) during treatment of sepsis and pneumonia with fucidin and methicillin has just been published (10) Results have been excellent and development of fucidin- and methicillin-resistant staphylococci has not occurred

The rather high incidence of tetracycline-resistant staphylococcus strains makes it inadvisable to employ the drug in combination with fucidin in the *initial treatment of severe and rapidly progressing* staphylococcal infections A combination of tetracycline and fucidin should not be used until resistance determination has proved that the strain isolated from the patient actually is sensitive to tetracycline

In the present work we have preferred to use fucidin to illustrate the value of combined therapy to prevent further development of resistant

staphylococci The facts applying to fucidin apply equally well to all other antibiotics even to those in which the long tail is absent The objection may be raised that sixteen years after the introduction of penicillin into the treatment of haemolytic streptococcal or pneumococcal infections no demonstrable increase in the resistance of these bacteria to penicillin has occurred But the same objection has been raised earlier in the case of gonococcal infections and yet now it is generally acknowledged that the resistance of gonococci to penicillin is not to be ignored With a view to protecting valuable drugs it can hardly be denied that combined therapy should preferably be used more widely than has hitherto been the case even in infections where treatment with one of the antibiotics has previously been adequate In chemotherapy it does not suffice to think only of the present—the future must also be considered

SUMMARY

One of the greatest drawbacks to chemotherapy is the emergence of chemo resistant bacteria

The rise in resistance takes place by selection of chemo resistant mutants during the treatment

Simultaneously a selection of the most virulent and the most infectious bacteria takes place as these bacteria have a greater possibility of spreading to other people and of causing diseases Consequently the rise in resistance will first occur in hospitals but may then spread to the population It is therefore of the greatest importance to try to prevent this rise in resistance

Using Fucidin® as an example it has been shown how this can be achieved Fucidin is a new Danish chemotherapeutic compound which is active against staphylococci and has proved to be very valuable in the treatment of staphylococcal infections

By means of a special technique it has been established that in all cultures of staphylococci some fucidin resistant mutants occur This is in agreement with the fact that when staphylococcal infections are treated with fucidin alone resistance to this compound may emerge and that during treatment of burns in a hospital fucidin resistant staphylococcal infections occurred in patients who had not been subjected to treatment with this compound in other words an incipient epidemic arose

Furthermore experiments have shown th[illegible] combination of fucidin [illegible]ycline in comparatively [illegible] In addition they show [illegible]

[illegible] introduced it would be [illegible]ertain the number of resistant mutants by means of the technique employed in this work and to establish which other chemo

therapeutic compounds are suitable for forming part of a combination to eliminate the said mutants

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FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF MICROBIOLOGY BERGEN NORWAY

SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

4 *Factor* h *Serum*

By

GUNNAR HAUKENES

Received 19 vi 63

The *h* antigen was characterized by *Oeding* (1953a) as heat stable and trypsin resistant apparently of polysaccharide nature Relatively few strains were found to agglutinate in the *h* serum unless examined also after autoclaving of the organisms

When pyogenic staphylococci were typed serologically cross reactions were commonly observed between the *a* *b* and *c* sera and the *h* serum when autoclaved organisms were used for agglutination This roused the present author s suspicion of the presence of a common heat st ... aled the ... s (5 6) ... to cause ... agglutination between the *b* and *h* factor sera

We have preferred to designate the above mentioned strong heat stable antigen of strain 17 A as the a_3 antigen since it has a near connection to the *a* antigens which is also in accordance with *Grun* s designation (4) The other agglutinins of factor *h* serum have been designated h_1 and *h*

Cohen & Oeding (1962) comparing the fluorescent antibody and the agglutination methods examined a *h* factor serum which was emptied for *a* agglutinins by absorption with strain 3647 and they demonstrated two distinct antibodies in the *h* serum

Recently *Oeding & Haukenes* (1963) have described a characteristic precipitation line in agar developing between *h* factor serum and strain 17 A Absorption and heat resistance experiments showed that the line was produced by the heat labile h_1 antigen and it was therefore designated the h_1 line

The present article deals with the antibody composition of the *h* factor serum as well as antibodies of strain 17 A immune serum

MATERIALS AND METHODS

Two new ...
antigen c ...
by phag ...

antibodies except c_1 (7), but without being agglutinated by the respective factor sera. The antigenic formula of this strain can therefore not be given until it has been settled whether these antigens are very weak or partly blocked or whether the absorption might be unspecific. The phage type of strain 5687 is 3A/3B/3C/55/71.

In addition to nutrient agar slants the phenol red mannitol salt agar introduced by *Chapman et al.* (1938) was used for cultivation. The mannitol salt agar used in our laboratory has the following composition:

Bacto Beef Extract	1 g
Proteose Peptone no. 3 (Difco)	10 g
Sodium chloride	7.5 g
D Mannitol	10 g
Agar agar	15 g
Phenol red	0.025 g
Distilled water	1000 g
pH adjusted to 7.4	

Slide agglutination with mannitol salt agar cultures was carried out by the same technique as for nutrient agar cultures. Most strains grown on mannitol salt agar gave stable suspensions in the controls but the suspensions were more granular than with nutrient agar cultures although easily distinguishable from true agglutination in the factor sera.

Otherwise the techniques were the same as in earlier studies (5, 6, 7).

EXPERIMENTAL AND RESULTS

Factor *h* serum is prepared by absorption of serum 17 A with strain 1503 (11). Theoretically a_2, a_5, and b_3 antibodies may be present in a 17 A immune serum in addition to *h* antibodies and antibodies which will be described in connection with the Cowan strains and strains 670 and 5687. Strain 1503 will only absorb antibodies to one of the Cowan factors.

The agglutinability of the type strains in two factor *h* sera which yield different agglutination patterns, is shown in Table 1. The results

TABLE 1

Agglutinability of the Type Strains in Two h Factor Sera

Strain	*h* Serum K 28		*h* Serum K 824	
	Live bacteria	Autoclaved bacteria	Live bacteria	Autoclaved bacteria
3647	—	++	+	++
F 21	—	—	(+)	—
17 A	+++	++	+(+)	+++
3189	—	—	(+)	—
2095	—	++	(+)	+++
Cowan I	—	—	+	(+)
Cowan II	—	(+)		—
Cowan III		++	+	+++
670	++	+	+	+
830	+++	++	+++	+++
5687	++	(+)	+	+
6376	—	—	(+)	—

Code (+) + ++ +++ Strength of agglutination
— No agglutination

The *h* factor serum was prepared by absorption of serum 17 A with strain 1503

are in accordance with *Oeding*'s findings (10) as the majority of the strains reacted only after autoclaving. Live bacteria of strain 17 A agglutinated more strongly in serum *h* K 728 than in serum *h* K 824 while strong reactions were obtained with autoclaved bacteria in both sera.

TABLE 2

Agglutinability of the Type Strains in the h *Factor Serum after Absorption with Type Strains*

Strain	*h* factor serum K 728 absorbed with					Unabsorbed
	2253	3647	17 A	670	5687	
17 A	+++	+++	—	+	++	+++
670	++	++	++	—	+	++
5687	++	++	++	—	—	++
830	++	—	—	++	—	+++

Code: See Table 1.

Portions of *h* serum K 728 were absorbed with each of the type strains (Table 2). Five different agglutination patterns were obtained indicating the presence of at least 3 distinct agglutinins. The re-absorbed sera were also tested with autoclaved bacteria (not included in the table) and the strains possessing the a_5 antigen agglutinated as in the re-absorbed *a b* and *ac* sera (5, 6, 7). In addition weak agglutinins to strains 670 and 5687 were demonstrated and they were not removed by absorption with the other a_5 strains. The agglutinability of autoclaved 670 and 5687 bacteria agreed with the reactivity of live cultures of the same strains, and the agglutinability of autoclaved bacteria of strains possessing the a_5 antigen agreed with the strong agglutination of live 830 bacteria. We need therefore not introduce more new antibodies to explain the reactions with autoclaved bacteria or the agglutination of live 830 bacteria.

TABLE 3

Factor h *Serum Absorbed with Strain 3647 and Re-absorbed with other Type Strains*

Strain	*h* Serum K 728 absorbed with strain 3647 and re-absorbed with				Not re-absorbed
	17 A	670	5687	Autoclaved 670	
17 A	—	—	++	++	+++
670	++	—	++	++	++
5687	++	—	—	—	++
Residual antibodies	h_2	None	h_1	h_1	$h_1 + h_2$

Code: See Table 1.

antibodies except c_1 (7) but without being agglutinated by the respective factor sera The antigenic formula of this strain can therefore not be given until it has been settled whether these antigens are very weak or partly blocked or whether the absorption might be unspecific The phage type of strain 5687 is 3A/3B/3C/55/71

In addition to nutrient agar slants the phenol red mannitol salt agar introduced by *Chapman et al* (1938) was used for cultivation The mannitol salt agar used in our laboratory has the following composition

Bacto Beef Extract	1 g
Proteose Peptone no 3 (Difco)	10 g
Sodium chloride	75 g
D Mannitol	10 g
Agar agar	15 g
Phenol red	0 025 g
Distilled water	1000 g
pH adjusted to 7 4	

Slide agglutination with mannitol salt agar cultures was carried out by the same technique as for nutrient agar cultures Most strains grown on mannitol salt agar gave stable suspensions in the controls but the suspensions were more granular than with nutrient agar cultures although easily distinguishable from true agglutination in the factor sera

Otherwise the techniques were the same as in earlier studies (5 6 7)

EXPERIMENTAL AND RESULTS

Factor *h* serum is prepared by absorption of serum 17 A with strain 1503 (11) Theoretically a_2 a_3, and b_3 antibodies may be present in a 17 A immune serum in addition to *h* antibodies and antibodies which will be described in connection with the Cowan strains and strains 670 and 5687 Strain 1503 will only absorb antibodies to one of the Cowan factors

The agglutinability of the type strains in two factor *h* sera which yield different agglutination patterns, is shown in Table 1 The results

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Agglutinability of the Type Strains in Two h *Factor Sera*

Strain	*h* Serum K 728		*h* Serum K 824	
	Live bacteria	Autoclaved bacteria	Live bacteria	Autoclaved bacteria
3647	—	++	+	++
F 21	—	—	(+)	—
17 A	+++	++	+(+)	+++
3189	—	—	(+)	—
2095	—	++	(+)	+++
Cowan I	—	—	+	(+)
Cowan II	—	(+)	—	—
Cowan III	—	++	+	+++
670	++	+	+	+
830	+++	++	+++	+++
5687	++	(+)	+	+
6376	—	—	(+)	—

Code (+) + ++ +++ Strength of agglutination
— No agglutination

The *h* factor serum was prepared by absorption of serum 17 A with strain 1503

are in accordance with *Oeding*'s findings (10) as the majority of the strains reacted only after autoclaving Live bacteria of strain 17 A agglutinated more strongly in serum *h* K 728 than in serum *h* K 824, while strong reactions were obtained with autoclaved bacteria in both sera

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	2253	3647	17 A	670	5687	
17 A	+++	+++	—	+	++	+++
670	++	++	++	—	+	++
5687	++	++	++	—	—	++
830	++	—	—	++	—	+++

Code See Table 1

Portions of *h* serum K 728 were absorbed with each of the type strains (Table 2) Five different agglutination patterns were obtained indicating the presence of at least 3 distinct agglutinins The re-absorbed sera were also tested with autoclaved bacteria (not included in the table), and the strains possessing the a_o antigen agglutinated as in the re absorbed *a*, *b*, and *ac* sera (5, 6, 7) In addition weak agglutinins to strains 670 and 5687 were demonstrated, and they were not removed by absorption with the other a_o strains The agglutinability of autoclaved 670 and 5687 bacteria agreed with the reactivity of live cultures of the same strains, and the agglutinability of autoclaved bacteria of strains possessing the a_o antigen agreed with the strong agglutination of live 830 bacteria We need therefore not introduce more new antibodies to explain the reactions with autoclaved bacteria or the agglutination of live 830 bacteria

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Factor h *Serum Absorbed with Strain 3647 and Re absorbed with other Type Strains*

Strain	*h* Serum K 728 absorbed with strain 3647 and re-absorbed with				Not re absorbed
	17 A	670	5687	Autoclaved 670	
17 A	—	—	++	++	+++
670	++	—	++	++	++
5687	++	—	—	—	++
Residual antibodies	h_2	None	h_1	h_1	$h_1 + h_2$

Code See Table 1

To facilitate the distinction of the other agglutinins of the h serum, a_5 antibodies were first removed by absorption with strain 3647 Thereafter portions of this serum were absorbed with each type strain The agglutination patterns yielded by these re-absorbed sera confirmed the presumption that only two different antibodies were present in addition to a_5 (Table 3) Strains 17 A and 670 possessed one common agglutinogen, which was heat-labile and has been designated h_1 Strain 670 has in addition one heat-stable antigen which is shared by strain 5687 and has been designated h_2 Since 17 A bacteria were used for production of the immune serum, this strain must also possess the h_2 antigen although apparently completely blocked in live nutrient agar cultures (cf also Table 2) Neither autoclaved bacteria of strain 17 A were agglutinated by h_2 agglutinins, but they were able to absorb the agglutinins More details of each antigen will be given in connection with the respective factor sera

Preparation of Individual Factor Sera

Strain 17 A has been found to have the antigens a_2, a_5, b_3, h_1, and h_2 as reported above and in earlier articles (5, 6), and in addition 4 new antigens which will be described later the Cowan factors 2 and 4, 670–1 (?), and 5687–1 Two 17 A immune sera were examined for their antibody composition, and antibodies to a_5, h_1, h_2, and Cowan factors 2 and 4 were demonstrated in different amounts No antibodies to the antigenic factors a_2, 5687–1, or 670–1 were found

Factor a_5 serum Theoretically absorption of serum 17 A with strain 670 should remove all antibodies except a_5 Strain 670 has, however, weak group antigens (*i e* polysaccharide A and antigen A (*Jensen*)), which will require great doses of bacteria for absorption Moreover, absorption with strain 670 leads to a lowering of the a_5 antibody titre even with standard doses, and absorption with greater doses will apparently leave poor residual a_5 antibodies It is therefore recommended to absorb first with strain 1503 and, if strong h_2 agglutinins, also with mannitol-salt agar cultures of strain Cowan I (cf below) Thereafter h_1 antibodies are removed by absorption with the smallest possible dose of 670 organisms The isolation of an h_1 strain which does not absorb a_5 antibodies will simplify these absorption procedures

One of our 17 A sera, K 824, had very weak h_1 and h_2 antibodies, but strong a_5 antibodies and was selected for preparation of the a_5 serum Thirty ml serum diluted 1 10 was absorbed with the growth of 10 Roux bottles of strain 1503 according to the standard technique Thereafter the growth of 5 Roux bottles of strain 670 was added to the serum, and after thorough shaking the mixture was centrifuged immediately

Of the type strains only strain 830 agglutinated in the a_5 serum when tested with live nutrient agar cultures, while both autoclaved bacteria and mannitol-salt agar cultures gave strong reactions with most a_5 strains (Table 4)

TABLE 4

Agglutinability of Various Cultures of the Type Strains in an a_5 Factor Serum

Strain	Nutrient agar cultures						Mannitol salt agar cultures			
	5 hours	18 hours	Auto-claved	with 3.6% salt	with 7.5% salt	with 1% mannit	6 hours	8 hours	18 hours	48 hours
3647	—	—	++	—	+	—	++	+++	++	+
17 A	—	—	++	—?	++	—	+++	+++	+++	+
2095	—	—	++	—	++	—	+++	+++	++	—
Cowan III	—	—	++	—	++	+	++	+++	++	+
830	++	++	++	++	++	++	+++	+++	+++	+
5687	—	—	—	—	(+)	—	++	(+)	—	—

Code See Table 1

The a_5 factor serum was prepared from serum 17 A K 824 by absorption with strains 1503 and 670

The influence of the growth time has been shown in Table 4 It appears that only 6-hour-old mannitol-salt agar cultures enabled the detection of the a_5 antigen in all strains Eighteen-hour-old cultures gave strong reactions with most strains, while the agglutinability was nearly lost after 48 hours Agglutination with nutrient agar cultures with mannitol added or with different salt concentrations shows that the increased agglutinability of mannitol-salt agar cultures primarily can be ascribed to their high sodium chloride content

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Factor h_2 serum Absorption of our K 728 17 A immune serum with the homologous strain left residual agglutinins to nutrient agar cultures of strains 670 and 5687 and also agglutinins to autoclaved bacteria of the same strains An identical factor serum was obtained when serum Cowan I was absorbed with the homologous strain, and a potent h_2 serum can also be prepared from serum 5687 as will be described in a forthcoming article These agglutinins could not be demonstrated in normal rabbit sera Thus the h_2 antigen appears to be completely blocked in some strains, and agglutination with mannitol salt agar cultures was therefore attempted (Table 5) Five strains now agglutinated in the h_2 serum i e 17 A, Cowan I, and Cowan II in addition to the two strains mentioned above The identity of the h_2 antigen in these strains was confirmed by absorption experiments, as mannitol-salt agar cultures exhausted the h_2 serum

The agglutinability of mannitol salt agar cultures was studied in

To facilitate the distinction of the other agglutinins of the *h* serum, a_5 antibodies were first removed by absorption with strain 3647 Thereafter portions of this serum were absorbed with each type strain The agglutination patterns yielded by these re-absorbed sera confirmed the presumption that only two different antibodies were present in addition to a_5 (Table 3) Strains 17 A and 670 possessed one common agglutinogen, which was heat-labile and has been designated h_1 Strain 670 has in addition one heat-stable antigen which is shared by strain 5687 and has been designated h_2 Since 17 A bacteria were used for production of the immune serum, this strain must also possess the h_2 antigen although apparently completely blocked in live nutrient agar cultures (cf also Table 2) Neither autoclaved bacteria of strain 17 A were agglutinated by h_2 agglutinins, but they were able to absorb the agglutinins More details of each antigen will be given in connection with the respective factor sera

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17 A	—	—	++	—?	++	—	+++	+++	+++	+
2095	—	—	++	—	++	—	+++	+++	++	—
Cowan III	—	—	++	—	++	+	++	+++	++	+
830	++	++	++	++	++	++	+++	+++	+++	+
5687	—	—	—	—	(+)	—	++	(+)	—	—

Code See Table 1
The a_5 factor serum was prepared from serum 17 A K 824 by absorption with strains 1503 and 670

The influence of the growth time has been shown in Table 4 It appears that only 6 hour old mannitol-salt agar cultures enabled the detection of the a_5 antigen in all strains Eighteen-hour old cultures gave strong reactions with most strains, while the agglutinability was nearly lost after 48 hours Agglutination with nutrient agar cultures with mannitol added or with different salt concentrations shows that the increased agglutinability of mannitol-salt agar cultures primarily can be ascribed to their high sodium chloride content

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17 A	—	—	++	—?	++	—	+++	+++	+++	+
209a	—	—	++	—	++	—	+++	+++	++	—
Cowan III	—	—	++	—	++	+	++	+++	++	+
830	++	++	++	++	++	++	+++	+++	+++	+
5687	—	—	—	—	(+)	—	++	(+)	—	—

Code See Table 1

The a_5 factor serum was prepared from serum 17 A K 824 by absorption with strains 1503 and 670

The influence of the growth time has been shown in Table 4 It appears that only 6-hour-old mannitol-salt agar cultures enabled the detection of the a_5 antigen in all strains Eighteen-hour-old cultures gave strong reactions with most strains, while the agglutinability was nearly lost after 48 hours Agglutination with nutrient agar cultures will [illegible]

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The agglutinability of mannitol-salt agar cultures was studied in

TABLE 5

Agglutinability of the Type Strains in an h_2 Factor Serum and Absorbing Capacity of Mannitol Salt Agar Cultures Grown at 20° C

Strain	Agglutinability in h serum			Absorbing capacity
	Nutrient agar	Mannitol agar 37° C	Mannitol agar 20°C	Mannitol agar cult 20 C
1503	—	—	++	+
2253	—	—	—	+
28	—	—	++	+
365	—	—	++	+
17 A	—	+	+++	+
Cowan I	—	++	++	+
Cowan II	—	++	++	+
670	++	++	++	+
5687	++	++	++	+
Other 9 type strains	—	—	—	—

Code for agglutination See Table 1
Under absorbing capacity + Absorption of h_2 agglutinins
— No absorption
The h_2 serum was prepared by absorption of serum 17 A with live 17 A bacteria

more detail with regard to the influence of the temperature and duration of growth It was found that agglutination with cultures grown at room temperature revealed the h_2 antigen in strains where it had been completely blocked when grown at 37° C The agglutinability of strain 28 at various conditions has been shown in Table 6 Strongest reactions were obtained with young cultures grown at 20° C Nutrient agar and blood agar cultures grown at room temperature or at 28° C agglutinated also very strongly, but they agglutinated often spontaneously and were less suitable for serological typing

TABLE 6

Agglutinability of Various Cultures of Strain 28 in an h_2 Factor Serum

Culture medium	Growth temperature	Age of culture			
		5 hours	12 hours	18 hours	48 hours
Mannitol agar	20° C			++	++
Nutrient agar	20° C			+++	+++
Blood agar	20° C			+	+++
Mannitol agar	28° C			++	+
Nutrient agar	28° C			(+)	(+)
Blood agar	28° C			+++	++
Mannitol agar	37° C	—		—	—
Nutrient agar	37° C	—		—	—
Blood agar	37° C			—	—

Code See Table 1
The h_2 factor serum was prepared by absorption of serum 17 A with strain 17 A
The growth after 5 and 12 hours at 20° C and 28° C was insufficient for agglutination

The absorbing capacity of the mannitol salt agar cultures grown at room temperature was also examined to confirm the identity of the h_2 antigen. The results are given in Table 5 together with the agglutinability of various cultures of the type strains. With the standard technique only two strains were found to have the h_2 antigen, while 9 strains appeared to possess the antigen when the absorbing capacity of mannitol salt agar cultures grown at room temperature was examined.

The Distribution of the h_1, h and a_3 Antigens among the Type Strains

The results reported above have been summarized in Table 7, which shows that as many as 13 out of 18 type strains possessed one or more of these antigens.

TABLE 7

Distribution of the h_1, h_2 and a_3 Antigens Among the Type Strains

Strain	Antigens		
1503		h_2	
2253		(h_2)	
28		h_2	
365		h	
3647			a_3
17 A	h_1	h_2	a_3
2095			a_3
Cowan I		h_2	
Cowan II		h_2	
Cowan III			a_3
670	h_1	h_2	
5687		h_2	a_3
830			a_3

Brackets denote that the antigen could only been demonstrated by absorption and not by agglutination.

Preparation of Individual Factor Sera

Factor a_3 serum: Absorption of serum 17 A with strains 1503 and 670 (for further details cf. above).

Factor h_1 serum: Absorption of serum 17 A with strains 1503 and 5687.

Factor h serum: Absorption of serum 17 A with strain 17 A or serum Cowan I with strain Cowan I.

DISCUSSION

At least three distinct antibodies were found in an h factor serum prepared by the standard method (11), and the corresponding antigens have been designated h_1, h_2 and a_3.

The h_1 antigen is heat labile and trypsin sensitive and may easily be

extracted from whole bacteria producing a characteristic line by agar precipitation

A seemingly pure h_1 serum has been prepared. Only two out of 18 type strains agglutinated in this serum, strains 17 A and 670, the latter being in addition selected as a type strain for the same reason. *Cohen & Oeding* (2) also found that very few strains reacted with their fluorescent h antibody, a reaction which seemed to be specific for the h_1 antibodies of the factor serum. *Hofstad* (8) examined 45 staphylococcal strains belonging to phage group I in various factor sera, and only 4 strains agglutinated in the h_1 serum. Thus the h_1 antigen appears to be less widely distributed than formerly believed (10). Prepared as described above the h_1 factor serum can now be used for serological typing of staphylococci employing live nutrient agar cultures, but more information about the occurrence of the h_1 antigen is necessary to realize the practical value of this factor serum.

The a_5 antigen is heat stable and trypsin resistant and partly blocked in live nutrient agar cultures. Both autoclaved bacteria and mannitol salt agar cultures unveiled the a_5 antigen, but the latter type of cultures gave somewhat stronger reactions and is to be preferred, also for practical reasons. The occurrence of the a_5 antigen among the type strains and earlier experience from typing of staphylococci in the h serum with autoclaved bacteria indicate that the a_5 antigen is widely distributed among pyogenic staphylococci. No direct correlation to phage types or groups is apparent, but immunologically the a_5 antigen seems to have more connection to the a antigens than to the h_1 and h_2 antigens. *Hofstad* (8) found that 36 out of 45 strains belonging to phage group I possessed the a_5 antigen. The a_5 antigen is probably also commonly found among strains belonging to phage group III (cf. (5) and (7)). A seemingly pure a_5 serum has been prepared yielding strong reactions with mannitol-salt agar cultures, and the serum will most probably be of considerable value in typing work.

Like the a_5 antigen the h_2 antigen appeared to be heat-stable and commonly blocked, in some strains blocked even in ordinary mannitol salt agar cultures. It is not clear whether this h_2 antigen is the same as that discribed by *Cohen & Oeding* (2). They used the fluorescent antibody staining technique and examined a number of staphylococcal strains in a h serum which had been prepared by absorbing serum 17 A with strains 1503 and 3647 and thus apparently containing no a_5 agglutinins. Of the type strains only strain 17 A reacted by this method (strain 670 was not included among the type strains at that time). Agglutination reactions indicated the presence of an additional antibody which for some reason seemed to have been lost or inactivated by the procedures for preparation of the conjugated antibody protein. Since agglutination with mannitol-salt agar cultures was not performed at that time, the distribution of the corresponding antigen among the type strains is not known. Most probably their h_2 antigen is the same as the

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The h_2 antigen is widely distributed among the type strains as 9 of 18 strains possessed the antigen when mannitol-salt agar cultures grown at 20° C were examined It may also be of interest that *Grun*'s strain A_4 (4) which is used for preparation of *l* factor serum, possessed the h_2 antigen *Hofstad* (8) used mannitol-salt agar cultures grown at 37° C and found that 24 out of 45 staphylococci from phage group I had the h_2 antigen A considerably greater number of strains will probably appear to possess the antigen when examined with cultures grown at 20° C

Oeding (1957) found that the agglutinability of his strains was lost when grown at room temperature It was therefore not unexpected to find that other antigenic structures were exposed at the bacterial surface under these conditions The type strains used by *Oeding* did not include 670 and 5687 which in the present study roused the author's suspicion of a new antigen that was completely blocked in the other strains The consequences of the present observations of blocked antigens for practical serological typing can not at present be fully surveyed Serological typing becomes still more complicated, but, on the other hand, a more precise immunological characterization of each strain is rendered possible Preliminary testing of the other factor sera with cultures grown at 20° C seems to indicate that it is primarily in connection with typing in h_2 factor serum and 670-1 factor serum (to be published later) that the 20° C cultures are superior to ordinary nutrient agar or mannitol-salt agar cultures The growth conditions have, however, to be subjected to more comprehensive investigations with regard to unmasking of antigens before definite conclusions can be drawn with regard to the ideal technique for practical serological typing

The nature of the antigen blocking is not fully understood It is evident that when the growth is retarded in some way by the addition of salt or by a lowering of the temperature, the heat-resistant antigens are exposed to the surface It may be hypothetized that the synthesis of protein antigens is retarded under these conditions, and that these antigens block the polysaccharide antigens If this holds true, and if we imagine the blocking merely to be of a mechanical value, at least two antigen layers may be postulated, an outer protein and an inner polysaccharide layer Such a proposition was set forth by *Oeding* in 1953 (9) based upon treatment of the agglutinogens with heat, trypsin, acid, alkali and alcohol *There is no* direct evidence for or against this view, as it is at present not known whether the blocking is due to a chemical linkage or to mechanical masking In the present investigations the h_2 antigen could not be demonstrated in strain 17 A nutrient agar cultures

extracted from whole bacteria producing a characteristic line by agar precipitation

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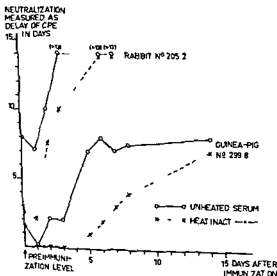
BRIEF REPORT

INFLUENCE OF HEAT INACTIVATION ON THE NEUTRALIZATION OF TYPE 7 ADENOVIRUS BY ANIMAL SERA

By *Gunvor Svartz Malmberg*

In certain human sera heat inactivation brings about a decrease in neutralizing capacity against type 7 adenovirus (1) The sequential appearance of different anti bodies after immunization of animals has been demonstrated with various antigens (2) The present report concerns the effect of heat inactivation on animal sera taken at different intervals after immunization with type 7 adenovirus

Freon 113 Rabbits were immunized by one single dose either 10 ml of undiluted antigen intravenously or 10 ml intramuscularly of antigen mixed with Freund's incomplete adjuvant Guineapigs were injected with 2 ml intramuscularly of an antigen adjuvant mixture followed by 1 ml of undiluted antigen intraperitoneally after 1 week Blood specimens were collected before immunization and afterwards four to six times during the first week and at the end of the second week Sera were separated by centrifugation within two hours after bleeding and stored at −85° C. Heat inactivation was carried out at 56° C for 30 minutes Neutralization tests were performed according to *Kjellen et al* (4) In this technique the time interval in days from the appearance of cytopathic changes in the virus control until such changes are detected in tubes inoculated with serum—virus mixture is used as a measure of the neutralizing capacity of the serum Unheated and heat inactivated samples from the same bleeding were tested simultaneously and so were as far as possible all specimens from one animal



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by agglutination That the antigen was present in these cultures is clear, however, since the immune serum which contained h_2 antibodies was produced from nutrient agar cultures

SUMMARY

Factor *h* serum appeared to be composed of antibodies to 3 different antigens·

1) h_1, which is heat-labile and produces a characteristic precipitation line in agar, and which seems to represent a rather uncommon finding among pyogenic staphylococci

2) h_2, which is heat-stable, widely distributed, and may require mannitol-salt agar cultures *grown at 20° C for detection*

3) a_s, which is also heat-stable and widely distributed and demonstrable by the use of mannitol-salt agar cultures grown at 37° C

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Annual Meeting November 29 and 30, 1963

Stockholm, Sweden

Meeting November 29–30

Microbial genetics. A symposium on molecular biology
(Arranged in collaboration with the Swedish Society for Microbiology.)

(*P. Reichard*, Chairman.)

A. *Survey Lectures*

Hultin, T. The Wennergren Institute, Stockholm University, Stockholm: THE METABOLISM OF CELLULAR INFORMATION.

Bertani, G. The Wallenberg Laboratory, Karolinska Institutet, Stockholm: RECENT FINDINGS OF BACTERIAL GENETICS IN HISTORICAL PERSPECTIVE.

Klein, G. Department of Tumor Biology, Karolinska Institutet, Stockholm: PROBLEMS CONCERNING GENETICS OF MAMMALIAN SOMATIC CELLS.

B. *Experimental Studies*

Boman, H. G. and *Gordon, J.* Institute of Biochemistry, University of Uppsala, Uppsala: IN VITRO METHYLATION OF RIBOSOMAL RNA.

The work reported is a continuation of previous studies on the methylation of S-RNA. We have isolated by differential centrifugation those (MS) particles that accumulate in the relaxed *E. coli* strain W6 during 60 min. starvation for methionine. The particle preparation is contaminated with some protein material. The particles contain 73 per cent ribosomal RNA (R-RNA), small amounts of S–RNA and 27 per cent protein. MS particles in sucrose gradients sediment like chloramphenicol (CM) particles. In the presence of ATP and a supernatant fraction methyl groups from methionine were incorporated into the RNA of MS particles (and also of CM particles). Cold S-adenosylmethionine quenches this incorporation. ^{14}C-methylgroups from S-adenosylmethionine were incorporated into RNA from MS particles with neither supernatant fraction nor ATP. The reaction product is RNase sensitive. Using phenol technique total RNA was prepared from a reaction mixture and fractionated by gel chromatography on Sephadex G 200. Both R-RNA and S-RNA were methylated and the incorporation was proportional to the amount of particles added. About 1 methylated base per 7000 bases was found as saturation value for R-RNA. ^{14}C-methylated nucleosides were isolated from *in vitro* labelled R-RNA and S-RNA. The chromatographic pattern corresponds with that from *in vivo* experiments.

The figure shows a typical curve from a rabbit and one from a guineapig Similar results were obtained with all of 5 rabbits and 7 guineapigs There were no essential differences whether or not adjuvant was used in the immunization of rabbits Heat inactivated preimmunization sera from a larger series of both species had as a rule little or no inhibitory capacity against adenovirus type 7 However unheated rabbit sera usually had considerable neutralizing effect whereas this occurred more seldom with guineapig sera Thus only among the guineapigs it was possible to choose animals with a low thermolabile inhibitory capacity for the experiments A rise in thermolabile inhibitory capacity was demonstrated within the first week after immunization in all guineapigs tested The heat inactivated samples usually did not reach the level of the unheated ones until the end of the second week The rabbits developed the same sequence but here the greatest differences between unheated and heat inactivated samples occurred on the first second and third day after immunization The peak level measurable by the neutralization test employed was reached also by the heat inactivated samples already on the third though sixth day

Rafajko (5) recently reported on early rise of thermostable antibodies in rabbits injected with adenovirus type 12 It must be considered as a possibility that the high thermolabile inhibitory capacity found in sera from normal rabbits might reflect an immunological status that is prepared for a more rapid response than would be expected by the negative finding of thermostable antibodies From the *results described it seems reasonable to conclude that type 7 adenovirus gives rise* in the host organism not only to thermostable antibodies but also to inhibitory factors dependent on or consisting of thermolabile components The further characterization of these factors requires investigations on sedimentability and dependence on complement activity as well as fractionation studies which are in progress

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Lagerkvist U Department of Pediatrics University of Gothenburg Gothenburg
SPECIES SPECIFICITY AND STRUCTURE OF TRANSFER RNA
To be published in J Mol Biol 1964

Hermodsson S Institute of Virology University of Uppsala Uppsala STUDIES ON SYNTHESIS OF INTERFERON IN VIRUS INFECTED CELLS

Varying amounts of interferon was produced in calf kidney cultures infected with different strains of parainfluenza virus type 3 (PIV 3) Infection with the strain 23B gave very low yields of interferon This strain was also able to inhibit the production of interferon induced by another virus and to inhibit the antiviral action of interferon It could not be shown that this phenomenon was due to inactivation of interferon in PIV 3 *infected cultures By the use of actinomycin* D which is considered to inhibit the RNA synthesis directed by the cell DNA evidence was obtained that the information for interferon synthesis is inherent in the cells It was also found that a UV irradiated suspension of the strain 23B induced an intense synthesis of interferon These results support the hypothesis that virus may actively inhibit the synthesis of interferon induced by the virus itself

Bertani F The Wallenberg Laboratory Karolinska Institutet Stockholm
OBSERVATIONS ON THE IMMUNITY OF LYSOGENIC BACTERIA
To be published in collaboration with Rene Thomas in Virology 1964

a) *Bacteriology*

Lindbom G Institute of Bacteriology University of Uppsala Uppsala
STAPHYLOCOCCAL INFECTIONS IN A THORACIC SURGERY UNIT

During a 1 year study 33 patients (10 6 per cent) acquired staphylococcal disease following surgery Fourteen infections were classified as serious contributing to the death of 7 patients In 9 cases the diagnosis of septicaemia was considered it was verified in 5 patients 4 of which died Phage typing revealed that only a narrow spectrum of staphylococci caused 2 or more infections 68 per cent of all infections were caused by strains either belonging to the 52/52A/80 81 complex or characterized by the 75/77 pattern Another 21 per cent were caused by staphylococci non typable both at R T D and 1000 × R T D

In the spring of 1961 there was an influx of virulent staphylococci from other parts of the hospital (80/KS6 75/77) At the same time there was a continuous presence of strains endemic to the ward among the personnel (80/81/KS6 81/KS6) The result was an outbreak of serious postoperative sepsis with a peak incidence of 36 per cent Closure of the unit changed the staphylococcal ecology and virulent staphylococci later grew uncommon

Approximately 2 3 of all infections were caused by strains that had been acquired after the operation Three separate chains of crossinfection could be identified Epidemiological data indicated that carriers among patients and personnel played a crucial role during the epidemic and *emphasized the risk of acquiring infections* during the early postoperative period

Hallan ler H O Institute of Bacteriology University of Uppsala Uppsala

FRACTIONATION OF STAPHYLOCOCCAL TOXINS

An evaporated culture supernate from staph aureus S6 c ntaining enterotoxin P fibrinolysin (staphylokinase) alpha haemolysin delta haemolysin hyaluronidase leucocidin l pase alkaline phosphatase was fractionated on cross linked dextrans (Sephadex) On Sephadex G-100 the material was separated into three main groups 1) l pase delta haemolysin alkaline phosphatase 2) hyaluronidase 3) fibrinolysin enterotoxin leucocidin alpha haemolysin Other types of Sephadex (G 200 G 75 G-50) gave no further separation With their help however the molecular weights were roughly calculated to 200 000 ab ut 50 000 and 10 000–50 000 respectively of the three groups

The antigenic composition of the fractions after separation on G 100 were followed by immuno-electrophoresis Lipase and delta haemolysin could be further separated on 2 5 per cent pearl condensed agar Delta haemolysin passed out into the void volume which in this case means an almost particular size of the substance (or at least 300 mμ) When 0 05 per cent Tween 80 was added to the buffer delta haemolys n passed into the network of the agar At the same time the recovery was about 350 per cent Enterotoxin B was further purified on the Cation exchanger Cellex p in a salt-grad ent from 0 01 M to 0 1 M potassium phosphate buffer pH 6 5 The enterotox n fraction was immunologically pure The activity recovery calculated from the beginning of the purification was about 55 per cent

Kjellander J and *Finland M* Thorn l ke Mem rial Laboratory Department of Medicine Harvard Medical School Boston Massachusetts U S A STAPHYLOCOCCUS ALBUS AS PRODUCER OF PENICILLINASE The essential *contents* of this paper have been published in Proc Soc Exp Biol Med *113* 1023 1037 1963

Dem ssie A Institute of Bacteriology University of Uppsala Uppsala THE ISOLATION OF ENTEROPATHOGENIC SALMONELLAE FROM THE RIVER FYRIS AND THEIR DETECTION BY THE FLUORESCENT ANTIBODY METHOD

Modern techniques for isolating salmonellae are efficient when applied to faeces With other materials such as well water river water and sewage systems complicated procedures are used

During a four months period May August 1963 an investigation aimed at isolating enteropathogenic salmonellae from the River Fyris was done *The results of a* modified swab technique (Moore) and filtration through membrane filter (Millipore HAWG 0 45 M) supercel and Seitz filter were compared

Negative results were obtained *when ampure river water was filtered through* membrane filter and Seitz filter Filtration through these filters was too slow and confluent overgrowth was noticed on Dc agar plates on successive plating

A total of 9 salmonellae strains were isolated One strain S typhimurium NS phage type was isolated by the super cel *method*

Swabs were made from strips of *gauze* compactly rolled round rectangular wire frames 15 × 20 cm and about 5 7 inches thick When such swabs are immersed in the river for from 48–96 *hours* they were effective traps for salmonellae

One strain of S *paratyphi B 3A1* var 2 one of S typh murium NS five of S blockley and one of S enteritidis were isolated by this method

After S. blockley had been isolated from the river it was possible to trace this strain to a manhole located near the home of the carrier by systematically investigating key manholes in the area.

Preliminary results with the fluorescent antibody method gave a threefold higher detection frequency than the conventional method.

Danielsson D. Institute of Bacteriology, University of Uppsala, Uppsala.
SEROLOGICAL STUDIES OF N. GONORRHOEAE AND OTHER NEISSERIA SPECIES BY MEANS OF THE DOUBLE DIFFUSION IN GEL TECHNIQUE AND THE FLUORESCENT ANTIBODY METHOD

The precipitinogenic relationship between N. gonorrhoeae strains and other neisseria species were studied by double diffusion in gel technique. The neisseria strains tested were treated by ultra sonic. For the comparative analyses a reference system was used containing 15 separate precipitating systems. The serum in this reference system was obtained from a rabbit immunized with formalin killed gonococci from the gonococcus reference strain. One or two of the precipitinogenic factors demonstrated in the reference strain were missing in some selected strains of N. gonorrhoeae. Meningococci belonging to the serological groups A, B, C and D were shown to have 11–12 antigenic factors and strains of N. sicca, N. catarrhalis, N. flava and N. flavescens 3–9 antigenic factors in common with the 15 factors demonstrated in the reference strain. It was further demonstrated that subculturing of the reference strain did not alter its precipitinogenic pattern.

The fluorescein isothiocyanate conjugated globulin of the reference serum gave a 3–4+ reaction with the reference strain at a maximal dilution of 1/256. A corresponding reaction was obtained with other gonococcal strains at dilutions of 1/64–1/128 and with meningococci at dilutions of 1/16–1/32. Some strains of the so called apathogenic neisseria species gave a weak 1–2+ reaction at a maximal dilution of 1/4–1/8. The precipitinogenic factors of the apathogenic neisseria strains in common with gonococci could not be correlated to the weak reactions obtained by the fluorescent antibody method. Preliminary results indicated that the majority of the antigenic factors demonstrated by the diffusion in gel technique is of intracellular origin, especially those in common for the different species within the neisseria group.

Holm S. and *Jonsson J.* Institute of Bacteriology, University of Gothenburg, Gothenburg.
EXPERIMENTAL STUDIES ON THE NEPHRITOGENIC EFFECT OF β-HEMOLYTIC STREPTOCOCCI IN RABBITS

A type 12 streptococcal strain isolated from a patient with acute glomerulonephritis and a type 3 (S 84) strain were tested for their nephritogenicity in rabbits. The streptococci were grown under stabilized pH conditions at pH 7.2 in a dialyzable medium. Four streptococcal preparations were produced from each strain: culture filtrates, washed streptococci killed with penicillin, washed streptococci treated with ultrasonic waves and dialysate from streptococcal cells. Each of the preparations from the two strains was injected intravenously into two rabbits and blood pressure, urine casts and urine protein were determinated. Histopathological examinations of the kidneys were performed 5–6 months after the first injection. All rabbits treated with type 12 streptococcal preparation developed hypertension and hematuria. Interstitial nephritis corresponding to Ellis type I was seen in two of these

rabbits. In the rabbits injected with type 3 streptococcal preparations no hypertension resulted but in two animals microscopic examination revealed tubular lesions and slight glomerular changes comparable to Ellis type II nephritis. All four rabbits injected with dialysate from streptococcal cells showed severe tubular damage and typical glomerulonephritis of Ellis type II.

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Philipson L. Institute of Virology, University of Uppsala, Uppsala. VIRUS ECLIPSE IN A CELL-FREE SYSTEM. To be published in Virology 1964.

Bengtsson S. and *Philipson L.* Institute of Virology, University of Uppsala, Uppsala. GEL FILTRATION OF ANIMAL VIRUSES ON PEARL CONDENSED AGAR.
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Mårtensson J. Institute of Bacteriology, Lund University, Lund. THE SYNTHESIS OF 7S γ GLOBULINS BY THE HUMAN FOETUS.
To be published in Acta path. et microbiol. scandinav. 1964.

Johansson B. Department of Medical Biochemistry, University of Gothenburg, Gothenburg. ISOLATION OF γ_{1A} GLOBULIN FROM HUMAN COLOSTRUM.

As shown by *Hanson* and *Johansson* human colostrum contains proteins which are closely related to γ globulin and γ_{1A} globulin in blood serum. The main part of this colostrum immune globulin fraction has an electrophoretic mobility corresponding to γ_{1A} globulin in serum. Colostrum γ_{1A} globulin has been isolated in small amounts by chromatography on DEAE-cellulose. For the isolation in larger scale the combination of column electrophoresis and gel filtration has been found preferable. The column electrophoresis was performed in a large scale apparatus as described by *Porath* with application of about 5 g of colostrum protein. The electrophoretic fraction containing γ_{1A} globulin was concentrated by ultrafiltration and filtrated through Sephadex G 200 for removal of small amounts of impurities.

The isolated protein was found to have a large molecular size when analyzed by thin layer gel filtration. This was not due to polymerization during the preparation

procedure as shown by comparative runs of unfractionated colostrum Determination of the N terminal amino acids with Sanger s method showed that glutamic acid aspartic acid occupied the N terminal positions in the protein together with small amounts of other amino acids The same N terminal amino acids have been found in γ globulin and γ_{1A} globulin from blood serum The presence of two types of poly peptide chains of different size was indicated by gel filtration after reduction of the protein with β mercaptoethanol This gel filtration was performed with a Sephadex gel having a water regain of 14 5 Quite similar results were obtained with serum γ globulin

Comparative analysis of the electrophoretic patterns of milk and serum γ_{1A} globulin have been made by starch gel electrophoresis After reduction of the proteins with β mercaptoethanol and electrophoresis of the reduced material in formate buffer containing β mercaptoethanol and 8 M urea the patterns of the milk and serum γ_{1A} globulin were not identical This might indicate a difference in structure between the two proteins

Moller G Department of Tumor Biology Karolinska Institutet Stockholm

EFFECT OF ISOANTIBODIES ON ERYTHROCYTES IN VIVO AND IN VITRO

Chromium 51 labelled erythrocytes were found to survive in adult histoincompatible recipients in spite of the fact that the hosts produced antibodies against the transfused cells Passive transfer of humoral antibodies into the recipients given incompatible erythrocytes caused a partial elimination of the foreign red cells The surviving fraction persisted as in the strain of origin however The red cells were found to be completely sensitive to the hemolytic effect of isoantibodies *in vitro* After contact with antibodies *in vivo* however the cells changed and they were completely resistant to hemolytic antibodies The cause of this change could be attributed to the contact with isoantibodies and to some host factors as well Decomplementation brought about by injection of heat aggregated human gamma globulin prevented efficiently the change in hemolytic sensitivity and the cells remained fully sensitive Treatment of red cells with antibodies and fresh mouse serum *in vitro* induced a state of resistance to the hemolytic effect of subsequently added guinea pig complement This did not occur unless mouse serum was present The findings suggested that the antigenic receptors of the red cells became blocked by isoantibodies and that the complement fixing sites of the antibodies were destroyed or blocked by some serum factor(s) in mice

Porath J Bennich H Faker H Killander J and Li N Institute of Biochemistry and Department of Clinical Chemistry University of Uppsala Uppsala

SEPARATION OF γ GLOBULINS AND THEIR CLEAVAGE PRODUCTS

To be published in Biochim Biophys Acta 1964

Hansson L A The Pediatric Clinic of the Karolinska Institute at Crown Princess Lovisa s *Children s* Hospital Stockholm ANTIBODY ACTIVITY IN URINARY 7S AND LOW MOLECULAR WEIGHT IMMUNOGLOBULINS

Several investigators have studied the relation of the antibody combining site to the structural subunits of γ globulin and have come to quite different conclusions One possible approach to this problem could be to study antibody activity demon

strable in concentrates of human urine as urinary immunoglobulins consist of 7S γ globulin some β_{2A} globulin as well as a considerable amount of a low molecular weight protein (γ_L) identical to L-chains of 7S γ globulin

The urinary 7S immunoglobulins were separated from the γ_L globulins by filtration through Sephadex G-100 and it was found that most antibody activity was localized in the 7S fraction (Anti blood group substance B anti T_2 phage and anti nuclear antibodies were employed) Most of the activity was related to the 7S γ-globulin but some to β_{2A} globulins as well However weak activity was also demonstrable in the γ_L fraction Density gradient ultracentrifugation of the γ_L fraction showed that the activity was present in material sedimenting similar as proteins of molecular weight from 13 000 to approximately 45 000 This γ_L fraction mainly contained L-chains but also some material related to H-chains The antibody activity of the γ_L fraction was lost if the H chain related material was eliminated by absorption with a specific anti H serum

Preliminary studies of the serum 7S anti T_2 antibodies with separation of the H and L-chains by gel filtration after dissociation of the 7S molecules with mercapto ethanol indicated that H and L-chains together may form the antibody combining site

Killander J Philipson L and *Bengtsson S* Department of Clinical Chemistry University Hospital and Institute of Virology University of Uppsala Uppsala SEPARATION OF RHEUMATOID FACTORS AND ANTISTREPTOLYSINS BY GEL FILTRATION ON AGAR AND DEXTRAN GELS

To be published in two papers

Killander J Bengtsson S and *Philipson L* FRACTIONATION OF HUMAN PLASMA MACROGLOBULINS BY GEL FILTRATION ON PEARL-CONDENSED AGAR Proc Soc Exp Biol Med 1964

Killander J and *Philipson L* SEPARATION OF RHEUMATOID FACTORS AND ANTISTREPTOLYSINS BY GEL FILTRATION AND PREPARATIVE ELECTROPHORESIS Acta path et microbiol scandinav 1964

Johansson S G O Hogman C F Killander J and *Wide L* Department of Clinical Chemistry and the Blood Transfusion Service University Hospital University of Uppsala Uppsala QUANTITATIVE DETERMINATION OF γ_{1M} γ_{1A} AND 7S γ GLOBULINS BY A MODIFIED BOYDEN TECHNIQUE

Human formaldehyde and tannic acid treated O Rh(D) negative red cells coated with 7S γ globulins were agglutinated by specific rabbit anti 7S γ globulin sera Addition of 7S γ globulins to the system inhibited the agglutination By comparison of the agglutination inhibition of serial 1 2 6 fold dilutions of the sample with a standard it was possible to estimate the concentration of 7S γ globulins in the sample Similar systems were set up for the determination of γ_{1M} and γ_{1A} globulins The preparations used as standards and for coating of cells were 7S γ globulins separated from pooled human plasma by Cohn fractionation and DEAE chromatography γ_{1M} globulins from normal sera and γ_{1A} globulins from myeloma sera separated by gel filtration and zone electrophoresis

Specific antisera were obtained by immunization of rabbits with purified immunoglobulins and absorption of the antisera against one immunoglobulin with solutions containing the others The specificity was checked by immunoelectrophoresis, double diffusion in agar gel and by agglutination of coated cells.

The 7S γ-globulin concentration in 19 normal sera were found to be 700–1200 mg per 100 ml The levels of γ_{1M}- and γ_{1A}-globulins were generally somewhat higher than has been reported with other methods The reason for this is being investigated

The standard deviations of the methods were 6–8 per cent. Concentrations in the order of magnitude 0 1–1 µg per ml could be detected

The methods have been of value in purification experiments with fractions containing minute amounts of immunoglobulins and in cases of hypogammaglobulinaemia and myeloma

Högman, C. F, *Killander, J* and *Johansson, S G O*, Department of Clinical Chemistry and the Blood Transfusion Service, University Hospital, University of Uppsala Uppsala: DETERMINATION OF IMMUNOGLOBULINS IN FRACTIONS CONTAINING PURIFIED ANTIBODIES

Rh(D) positive and Rh(D) negative red cells incubated with whole serum or antibody-containing fractions obtained by gel filtration on Sephadex G–200 were used in antiglobulin consumption studies After thorough washing the cells were incubated with specific rabbit anti-γ_{1M}, anti-γ_{1A} and anti 7S γ-globulins Remaining antiglobulin was determined by agglutination of formaldehyde and tannic acid treated red cells coated with purified γ_{1M} γ_{1A} and 7S γ-globulin preparations Anti γ_{1M} and anti-7S γ-globulin but not anti-γ_{1A}-globulin were consumed by the sensitized Rh-positive red cells indicating fixation of γ_{1M} and 7S γ-globulin Rh-antibodies to the original red cells

Eluates were made from Rh positive and Rh negative cells previously being incubated with Rh antibodies The concentration of 7S γ-globulins in the eluates was determined by a haemagglutination inhibition technique (*Johansson et al* Acta path et microbiol scandinav 1964) and compared with the titre of Rh antibody tested as agglutination of papain treated cells in saline In most of the experiments 7S γ-globulin was detected only in the Rh positive cell eluates Absorption studies of these eluates with Rh positive and Rh negative cells further indicated that the globulin consisted mainly of Rh antibody protein The concentration of 7S γ-globulin was 1 8 µg per ml in most experiments and the titres of antibody was 4–25 The calculated concentration of 7S γ-globulin in one agglutinating dose was 0 1–0 5 µg per ml

FROM THE INSTITUTE OF GENERAL ZOOLOGY UNIVERSITY OF COPENHAGEN
AND THE DEPARTMENT OF ORAL PATHOLOGY THE ROYAL DENTAL COLLEGE
AND THE DENTAL DEPARTMENT THE UNIVERSITY HOSPITAL COPENHAGEN

STUDIES IN ORAL LEUKOPLAKIAS

6 *Deposits of Amyloid in the Oral Submucosa Induced by Prolonged Use of Snuff*

By

HANS LYON H L POULSEN and J J PINDBORG

Received 19 vi 63

In a preliminary report *Pindborg & Poulsen* (10) described a peculiar change in the connective tissue underlying oral leukoplakias induced by the prolonged use of snuff

Seven patients—all men—had for periods varying from 20 to 50 years used snuff (of the brand called Gothenburg snuff) placing the quid of snuff in the labial groove corresponding to the lower incisors Here the mucous membrane was slightly whitish and had a delicately folded appearance

The tissue was fixed in formalin and embedded in paraffin The histologic investigation (10, 11) reveals a thickening of the squamous epithelium and, in 4 of the patients, areas of homogeneous tissue in the underlying connective tissue The thickening of the squamous epithelium is accounted for by a peculiar parakeratosis of the superficial layers with big clear cells

This study is planned as an attempt to define more closely the chemical composition of the above mentioned homogeneous tissue based upon the 4 patients mentioned above plus 3 more patients

METHODS

The following methods were applied to the sections

A *Histological Methods*

1 Haematoxylin and eosin technique
2 Van Gieson and Hansen s method

B *Histochemical Methods*

3 Periodic acid Schiff technique (PAS) according to *McManus & Mowry* (8)
4 Amylase hydrolysis using the method of *Lillie & Greco* (6) followed by the periodic acid Schiff technique

Supported by a research grant DE-1358 from the *National Institute of Dental Research National Institutes of Health U S Public Health Service*

Specific antisera were obtained by immunization of rabbits with purified immunoglobulins and absorption of the antisera against one immunoglobulin with solutions containing the others The specificity was checked by immunoelectrophoresis double diffusion in agar gel and by agglutination of coated cells

The 7S γ globulin concentration in 19 normal sera were found to be 700–1200 mg per 100 ml The levels of γ_{1M} and γ_{1A} globulins were generally somewhat higher than has been reported with other methods The reason for this is being investigated

The standard deviations of the methods were 6 8 per cent Concentrations in the order of magnitude 0 1–1 µg per ml could be detected

The methods have been of value in purification experiments with fractions containing minute amounts of immunoglobulins and in cases of hypogammaglobulinaemia and myeloma

Hogman C F Killander J and *Johansson S G O* Department of Clinical Chemistry and the Blood Transfusion Service University Hospital University of Uppsala Uppsala DETERMINATION OF IMMUNOGLOBULINS IN FRACTIONS CONTAINING PURIFIED ANTIBODIES

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TABLE 1

Summary of Results with Histochemical and Metachromatic Methods

	15306	15219	15281	15524	15589	15595	15865
Periodic acid/Schiff	++++	++++	+++	++++	++++	++++	++++
Amylase + Periodic acid Schiff	++++	++++		++++	++++	++++	++++
Alcian blue	0			0	0	0	0
Sulphation + Alcian blue		0		0	0	0	0
Ninhydrin/Schiff		+++	++	+++	+++	+++	+++
Dinitrofluorobenzene			+		++	++	++
Crystalviolet		meta chromat	meta chromat	meta chromat	meta-chromat	meta chromat	meta chromat
Toluidine blue 0.01%				ortochr	ortochr	ortochr	ortochr
Toluidine blue 3%				ortochr	ortochr	ortochr	ortochr
Sulphation + Toluidine blue				ortochr	ortochr	ortochr	ortochr

++++ very strong reaction   +++ strong reaction   ++ moderate reaction   + weak reaction   0 no reaction

5 Alcian Blue method according to *Ison* (7)
6 Sulphation performed according to *Lewis & Grillo* (5) followed by the Alcian Blue method
7 Ninhydrin/Schiff test according to *Burstone* (2)
8 Dinitrofluorobenzene method (DNFB) according to *Burstone* (2)

C *Metachromatic Methods*
9 Crystal violet according to *McManus & Mowry* (8)
10 Toluidine blue in a 0 01 per cent aqueous solution according to *McManus & Mowry* (8) and in a 3 per cent aqueous solution buffered to pH 2 9 at 70° C according to *Larsen* (4)
11 Sulphation performed according to *Lewis & Grillo* (5) followed by toluidine blue in 0 01 per cent aqueous solution

RESULTS AND DISCUSSION

The results are summarized in Table 1

A *Histologic Findings*

The homogeneous tissue is seen at the transition between lamina propria and tela submucosa either as an approximately 150 μm thick band parallel to the surface of the epithelium or as small areas in connection with the alveoli of the salivary glands This tissue is acidophilic with (1) haematoxylin and eosin and yellow with (2) van Gieson and Hansen's stain

B *Histochemical Reactions*

3 The subepithelial masses react strongly with Schiff s reagent after oxidation with periodic acid in the PAS test As this test demonstrates 1,2 glycol groups or the equivalent amino or alkylamine derivatives (*Hale* (3)), it may safely be concluded, that the homogeneous tissue at least in part consists of polysaccharides other compounds containing these groups being dissolved by the preceding treatment

4 Treatment of the slides with amylase does not alter the periodic acid/Schiff reaction of the subepithelial masses

5 The homogeneous tissue is not stained by the Alcian Blue method According to *Pearse* (9) acid mucopolysaccharides of epithelial and connective tissue mucin are stained, while the majority of mucoproteins are not stained by the use of short staining

6 After preceding sulphation of the sections the Alcian blue reaction of the homogeneous tissue is still negative The introduction of sulphate ester groups by sulphation may make the staining of polysaccharides possible or greatly increase the intensity of the staining The negative reaction of the subepithelial deposits may be explained either by the carbohydrate in these being dissolved by the sulphuric acid or by a too low surface density of hydroxyl groups on the carbohydrate to give sufficient anionic groups after the sulphation for linkage with the dye

7 The homogeneous masses give a moderately strong reaction with the ninhydrin/Schiff method This demonstrates aldehydes produced

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Ninhydrin/Schiff			+++	++	+++	+++	+++	+++
Dinitrofluorobenzene				+		++	++	++
Crystalviolet			meta-chromat	meta-chromat	meta-chromat	meta-chromat	meta-chromat	meta-chromat
Toluidine blue	0.01%				ortochr	ortochr	ortochr	ortochr
	3%				ortochr	ortochr	ortochr.	ortochr
Sulphation + Toluidine blue					ortochr	ortochr	ortochr	ortochr

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6. Sulphation performed according to *Lewis & Grillo* (5) followed by the Alcian Blue method
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C. *Metachromatic Methods*

9. Crystal violet according to *McManus & Mowry* (8)
10. Toluidine blue in a 0.01 per cent aqueous solution according to *McManus & Mowry* (8) and in a 3 per cent aqueous solution buffered to pH 2.9 at 70° C according to *Larsen* (4)
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7. The homogeneous masses give a moderately strong reaction with the ninhydrin/Schiff method. This demonstrates aldehydes produced

UNIVERSITY INSTITUTE FOR EXPERIMENTAL MEDICINE, NØRRE ALLÉ 71, COPENHAGEN, DENMARK

MECHANISM OF THE INCREASED SENSITIVITY TO RENIN IN NEPHRECTOMIZED ANIMALS

By

JENS BING

Received 30.vi.63

Tigerstedt & Bergman (1898) found that nephrectomized animals react with both an increased and a prolonged pressor response to injections of renin. This finding has been confirmed by many investigators, but the mechanism of the potentiation is still unknown. Table 1 gives the essential proposed explanations of the potentiation, the reasons why most of them have now been discarded and references to the literature. The last explanation given in the table, according to which the potentiation is due to an activator of the renin angiotensinogen system could so far be valid for the results of some recently published studies (*Bing & Magill* (1963)), as they showed that cross circulation between a normal and a nephrectomized rat results in a potentiation of the pressor response to renin in both animals. In accordance with this incubation of plasma from both animals with renin gave increased angiotensin yields. Similarly a potentiation of the response to renin was found after intravenous injection of about 2 ml plasma from nephrectomized rats or guinea pigs in normal rats, these results confirming previous studies by *Blaquier et al.* (1962). The potentiation phenomenon must thus be related to an activator or some other humoral principle(s). The present experiments were undertaken with the aim of studying the nature of this (these) humoral principle(s).

MATERIAL AND METHODS

furthermore give a metachromatic reaction with crystal violet it is concluded that they in all essentials are identical with amyloid

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NOVO RESEARCH INSTITUTE FOR EXPERIMENTAL MEDICINE, NORRE ALLE 71
COPENHAGEN DENMARK

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MATERIAL AND METHODS

Female albino rats of about 200 grams, guinea pigs of either sex weighing from 600 to 1100 grams and guenon monkeys of about 3 kg were used.

Determination of the change in angiotensin formation by addition of plasma from normal or nephrectomized animals was performed by adding 1 ml plasma to 2 ml of a horse angiotensinogen preparation. After mixing they were preheated to 37° C

Supported by grants from *King Christian X's Foundation* and *Fonden til Lægevidenskabens Fremme*. The author is indebted to the *Nordisk Insulinlaboratorium* for the human renin, to *Ferrosan* for the hog renin and horse angiotensinogen, to *Ciba* for synthetic angiotensin, to *Leo* for heparin and penicillin, to the blood bank of the *University Hospital, Copenhagen* for human plasma, and to *Herdis von Magnus* M.D., *The State Serum Institute* for the monkeys.

TABLE 1

Proposed explanation	Previous results	References
Unspecific increase in sensitivity of the vessels	Discarded because renin gives both increased and prolonged pressor response With other pressor substances one or both of these two changes are missing the only exception being vasopressin the potentiation of which may be due to a special mechanism	*Renin* 23 18 13 14 10 3 *Angiotensin* 13 15 7 3 2 *Adrenalin and Noradrenalin* 13 15 7 8 3 *Tryptamin and Serotonin* 15 *Barium Chloride* 15 *Gynergen* * *Vasopressin* 15 7 11 8
Reduction of the vascular bed or lack of renal destruction of renin or/and angiotensin	Discarded because the potentiation A) does not appear until about 4 hours after nephrectomy and B) is uninfluenced by administration of renin through the renal artery of a freshly excised kidney	A) 16 18 14 7 3 B) 3
Increase in blood or extracellular fluid volume	Discarded because the response to renin is normal after ureter transplantation into the vena cava	15
Lack of renal angiotensinase	Discarded because the sensitivity to angiotensin is uninfluenced by nephrectomy	17 7 10 3 2
Increase in amount of converting enzyme	Unproved	19
Increase in amount of angiotensinogen	Differing results some finding increased angiotensinogen in plasma (A) others showing that plasma from nephrectomized animals also potentiates a system containing excess of angiotensinogen (B)	A 18 17 B C 19 3 2
Loss of an inhibitor of the renin angiotensinogen system	Reduction of the increased angiotensin formation in plasma of nephrectomized by addition of (*in vitro* exp) or cross circulation with normal plasma (A) not confirmed in recent experiments (B)	A 18 15 19 10 5 B 2
Increase in an activator of the renin angiotensinogen system	Increased angiotensin formation by addition of (*in vitro* experiment) or either cross circulation with or injection of plasma from nephrectomized animals	3 2

The table gives 1) the previously proposed explanations of the increased sensitivity to renin in nephrectomized animals 2) the results of experiments showing that most of these explanations are *untenable and* 3) *references to the literature* the numbers referring to the list at the end of this paper The reference marked * refers to unpublished experiments of the present author

and further 1 ml of a similarly preheated hog renin preparation diluted with physiological salt solution or phosphate buffer (pH 7 5) was added The mixture was incubated for 10 min at 37° C boiled centrifuged and the supernatant tested for angiotensin Angiotensinase was destroyed before testing both in the angiotensinogen preparation and in the plasma samples by subjecting them to a pH of 3 5 at 25° C

angiotensin (Hypertensin Ciba) for 10 minutes With this standard technique angiotensinogen was in excess This was shown both by using two times the angio

containing from 0 1 to 2 5 Dog Units Renin 2) In studies on the influence of changes in angiotensinogen concentration (Fig 6) 0 5 ml of undiluted or 50 or 20 per cent plasma in 0 9 per cent saline was incubated with 3 ml buffer and 0 5 ml of a renin solution containing 2 5 Dog Units Renin 3) In studies on the relation between angiotensin yield and angiotensinogen concentration (Fig 7) the yield was determined after incubation of 0 5 ml plasma with 3 ml buffer and 0 5 ml of a renin solution containing 0

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determination of the pressor response to intravenous injection of 0 05 to 0 20 ml of the preparations in female rats weighing about 180 200 grams anaesthetized with amytal and pretreated with either 0 005 mg ergotamine tartrate or pentolinium 3 mg kg The pressor responses were compared with those obtained after injection of 1 to 10 ng synthetic angiotensin

RESULTS

1 *Potentiation of Angiotensin Formation by Addition of Plasma to a Renin Angiotensinogen System Containing an Excess of Angiotensinogen*

While addition of plasma from normal rats had no or only slight effect on the angiotensin formation in the standard angiotensinogen renin system containing an excess of antigotensinogen plasma from *nephrectomized rats* increased the yield to from 2 to 4 times the standard yield, (Fig 1), these results being in accord with those of *Regoli et al* (1961) and *Blaquier et al* (1962)

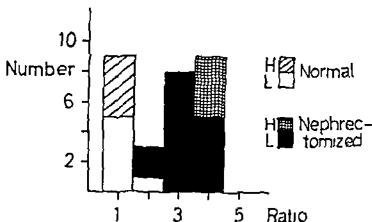


Fig 1

The potentiation of angiotensin formation in the *horse angiotensinogen hog renin system* is given by help of the ratio between the yields obtained by the following two incubations

$$\frac{\textit{Angiotensinogen + plasma + renin}}{\text{Angiotensinogen + saline + renin}}$$

the amount of angiotensinogen being in excess The values are given for plasma from *normal and nephrectomized rats* of two different strains H and L The yield with plasma from nephrectomized is seen to be 3 to 4 times that obtained with plasma from normal rats

Contrary to normal rat plasma, plasma from normal *guinea-pigs* was found to give marked increase from 2 to 6 times the normal yield (Fig 2) In accordance with this plasma from nephrectomized *guinea pigs* gave further increase, the angiotensin formation here being 5 to 9 times the normal yield

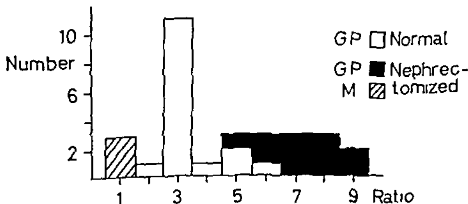


Fig 2

The potentiation of angiotensin formation in the *horse angiotensinogen hog renin system* is given by help of the ratio used in Fig 1 While addition of plasma from *nephrectomized monkeys* (M) is without effect plasma from *normal and nephrectomized guinea pigs* (GP) increases the yield 2 to 6 and 5 to 9 times respectively

2 Species Specificity of the Angiotensin Formation Promoting Factor

After having seen that addition of plasma from nephrectomized rats and from normal and nephrectomized guinea pigs to a hog renin horse angiotensinogen system increases the yield of angiotensin similar experiments with addition of rat and guinea pig plasma to a human renin monkey or human angiotensinogen system were performed As was the case with the hog renin horse angiotensinogen system the human monkey system contained an excess of angiotensinogen

TABLE 2

Renin	Angiotensinogen	Potentiating effect of plasma from nephrectomized		
		Guinea pigs	Rats	Monkeys
Hog	Horse	+	+	0
Human	Monkey or Human	+	0	+

The species specificity of "the potentiating factor is the same as that of angiotensinogen hog renin producing angiotensin with plasma from both guinea pigs and rats but not with plasma from monkeys and human renin forming angiotensin with plasma from both guinea pigs and monkeys but not with rat plasma

As shown in Table 2 the experiments showed that while plasma from both normal and nephrectomized guinea pigs increases the angiotensin formation as well in the human human system (Fig 3) as in the hog horse system (Fig 2) plasma from nephrectomized rats which has a marked effect on the hog horse system (Fig 1) has no potentiating effect on the human renin human angiotensinogen system (Fig 3)

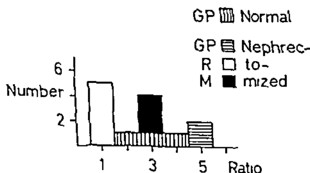


Fig 3

The potentiation of angiotensin formation in the human or monkey angiotensinogen human renin system is given by ...

... without effect in the hog horse system (Fig 2) ... nephrectomized monkeys (M) which was ... potentiated the human renin monkey angiotensinogen system giving a ratio of about 2 5

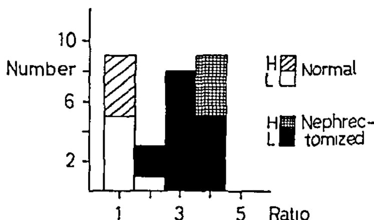


Fig 1

The potentiation of angiotensin formation in the *horse angiotensinogen-hog renin system* is given by help of the ratio between the yields obtained by the following two incubations:

$$\frac{\text{Angiotensinogen} + \text{plasma} + \text{renin}}{\text{Angiotensinogen} + \text{saline} + \text{renin},}$$

the amount of angiotensinogen being in excess The values are given for plasma from *normal and nephrectomized rats* of two different strains H and L The yield with plasma from nephrectomized is seen to be 3 to 4 times that obtained with plasma from normal rats

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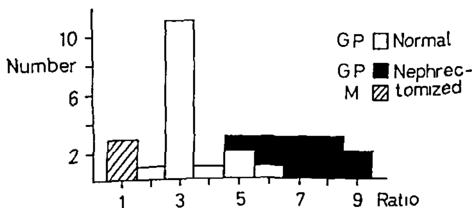


Fig 2

The potentiation of angiotensin formation in the *horse angiotensinogen-hog renin system* is given by help of the ratio used in Fig 1 While addition of plasma from *neprectomized monkeys* (M) is without effect plasma from *normal and nephrectomized guinea pigs* (G P) increases the yield 2 to 6 and 5 to 9 times respectively

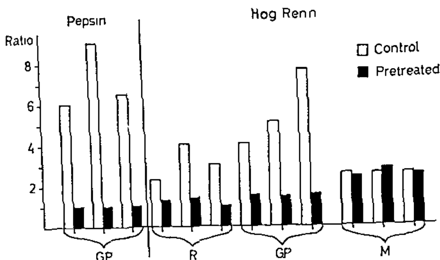


Fig 4

The effect of pretreatment with pepsin or hog renin on the factor potentiating angiotensin formation in plasma from nephrectomized animals The potentiation is given by help of the ratio used in Fig 1 Pretreatment with *pepsin* results in total inactivation of "the factor Pretreatment with *hog ren n* results in subtotal to total inactivation in rat (R) and guinea pig (G P) plasma but is without effect on monkey (M) plasma the ability of hog renin to inactivate the factor thus being identical with its ability to split angiotens nogens in plasma from these three species

obtained in experiments in which plasma from nephrectomized rats and guinea pigs was pretreated by incubation with semipurified hog renin and dialysed as this pretreatment was found to remove the potentiating effect on the angiotensinogen renin system As the renin is not pure the destruction might be due to an other enzyme in the preparation but this explanation was made unlikely, as it was found that similar incubation with the same hog renin preparation did not destroy the potentiating factor in plasma from nephrectomized monkeys (Fig 4)

4 *Relation between the Angiotensin Yield in a System Containing an Excess of Angiotensinogen and the Angiotensinogen Concentration*

The previous experiments were all performed in a renin angiotensinogen system containing an excess of angiotensinogen (see "Methods) As it is known that enzyme catalysed processes depend other things being equal upon the concentration of the enzyme and of its substrate the following studies on the influence of these concentrations were performed

The influence of changes in renin concentration was studies in experiments (Fig 5) in which constant amounts of plasma from normal or either 6 or 24 hours nephrectomized rats were incubated with a constant amount of a buffer (pH 7 5) and a constant volume of a renin

Similarly it was found, that plasma from nephrectomized monkeys had a potentiating effect on the angiotensin yield in the human renin-monkey angiotensinogen system (Fig 3) but was without effect when added to the hog renin-horse angiotensinogen system (Fig 2) The species specificity of the potentiating factor in plasma from nephrectomized animals is thus identical with that previously found in angiotensinogen (*Braun—Menendez et al* 1946), which so far were confirmed in the present studies, as hog renin was found to react with guinea pig and rat, but not with monkey and human angiotensinogen, while human renin reacted with human, monkey and guinea pig, but not with rat angiotensinogen This opens the possibility that the potentiation of the angiotensin formation in the renin-angiotensinogen plasma system is due to addition of angiotensinogen from plasma of nephrectomized animals

TABLE 3

	Angiotensinogen	»Potentiating factor«
Species specificity	+	+
Dialysable	0	0
Supernatant after Dialysis	+	+
Euglobulin Fraction	0	0
Destruction by heating to 56° C	+	+
Destruction by Pepsin	+	+
Destruction by hog renin in plasma from nephrectomized rats and guinea pigs	+	+
Destruction by hog renin in plasma from nephrectomized monkeys	0	0

Similarities between angiotensinogen and the "factor" found in plasma of nephrectomized animals potentiating the renin angiotensinogen system

3 *Similarities in Physical and Chemical Characteristics between the Potentiating Factor and Angiotensinogen*

In order to test the similarities between the potentiating factor and normal angiotensinogen, comparison of some of their physical and chemical characteristics were performed (Table 3) The table shows that besides having the same species specificity both are non dialysable, and after dialysis at a pH of 5 3 both the total amount of angiotensinogen and the total amount of potentiating factor are found in the supernatant, while the precipitated euglobulins do not contain any of these factors

It was also found that heating the plasma to 56° C for half an hour partly or totally destroys both angiotensinogen and the potentiating factor As these physical and chemical similarities are rather unspecific, being common for many substances, it is of more interest that the potentiating factor in plasma from nephrectomized rats and guinea-pigs in the same way as angiotensinogen from normal plasma is destroyed by incubation with pepsin Still more convincing results were

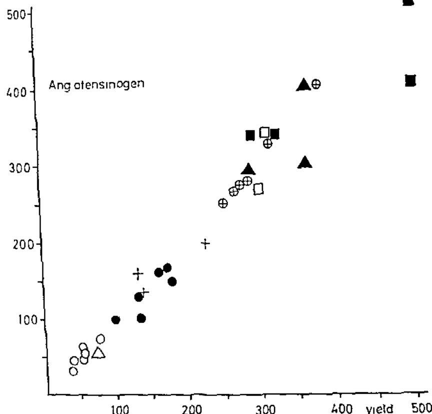


Fig 7

Relation between angiotensin yield and angiotensinogen concentration Both values being given in nm angiotensin There is a good correlation between the two values making it highly probable that increases in yield are caused by increases in angiotensinogen concentration Values are given for normal rats (marked ○) and for rats 3 (△) 6 to 8 (●) 9 to 11 (⊕) 12 (□) 18 (▲) and 24 hours (■) after nephrectomy Values from mixtures of plasma samples are marked +
Further details of the incubation are given in Methods

solution containing from 0 1 to 2 5 Dog Units renin (Nutr Bioch Corp) In this way it was found that the angiotensin yield was increasing with increasing amounts of renin a maximal value being reached with plasma from both normal and 6 hours nephrectomized rats, while the values with plasma from 24 hours nephrectomized rats did not reach a plateau The figure at the same time shows a very clear difference between the results obtained with the three plasma the difference between the yield obtained with plasma from normal and 24 hours nephrectomized rats being higher about 1 to 10 in this system than in the standard system used in the previous experiments

The influence of changes in angiotensinogen concentration was similarly studied in plasma from both normal and 6 or 24 hours nephrec

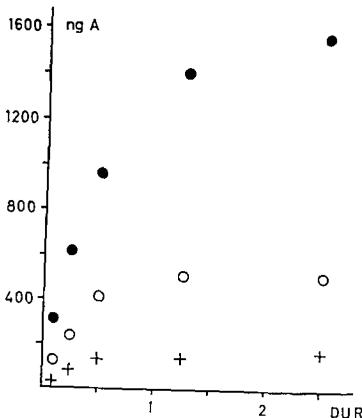


Fig 5

Influence of changes in renin concentration The angiotensin yield given in ng angiotensin (ngA) is increasing with increasing amounts of renin the values of which are given in Dog Units (D U R) While a maximal value is reached with plasma from both normal (marked +) and 6 hours nephrectomized rats (marked ○) the values with 24 hours nephrectomized rats (marked ●) do not reach a plateau Further details of the incubation are given in "Methods"

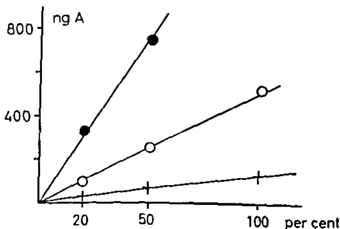


Fig 6

Influence of changes in angiotensinogen concentration The angiotensin yield given in ng angiotensin (ngA) varies with the concentration of plasma (angiotensinogen) given in per cent of undiluted plasma Values are given for experiments with plasma from both normal (marked +) 6 hours nephrectomized (marked ○) and 24 hours nephrectomized rats (marked ●)

Further details of the incubation are given in "Methods"

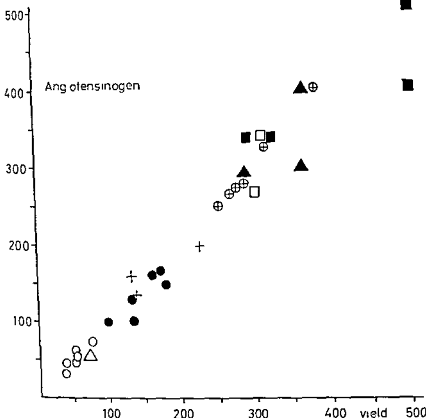


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The influence of changes in angiotensinogen concentration was similarly studied in plasma from both normal and 6 or 24 hours nephrec

tomized rats, the determination being made in a system in which renin was in excess. Using different concentrations of each plasma it was found (Fig. 6) that the values obtained with plasma from 6 and 24 hours nephrectomized rats follow other lines than the values of the normal rats. The figure also shows that in each of these 3 groups the angiotensin yield varies very closely with the changes in concentration of plasma.

Relation between angiotensin yield and angiotensinogen concentration. Having seen that both the angiotensin yield (obtained in a system in which angiotensinogen is in excess) and the total amount of angiotensinogen (determined in a system in which renin is in excess) are increasing with the time from nephrectomy it was of interest to compare these two values in plasma obtained at different times after nephrectomy. The result of such experiments are given in Fig. 7, showing a close correlation between angiotensin yield and angiotensinogen concentration. The same correlation was found in mixtures of plasma from normal and nephrectomized animals (Fig. 7). In experiments, in which the angiotensin yield and the angiotensinogen concentration were determined in mixtures of equal parts of plasma from normal and nephrectomized rats, the values obtained were about the half of the sum of the values in the two plasma samples. The figure further shows, how the plasma angiotensinogen concentration is increasing in the first 9 to 12 hours after nephrectomy, after which time rather constant values of about 8 to 10 times the normal concentration are obtained.

DISCUSSION

In the first parts of the present studies it is shown, that the humoral factor causing both an increased sensitivity to renin in nephrectomized animals and an increased angiotensin yield in incubation experiments with their plasma (Fig. 1, 2 and 3) has so many characteristics in common with angiotensinogen (Tables 2 and 3), that it is highly probable that it is itself an angiotensinogen. As the increased angiotensin yield is obtained by addition of plasma from nephrectomized animals to a renin-angiotensinogen system, containing an excess of angiotensinogen, it was first believed that the change was due to the presence of qualitative changed angiotensinogen(s) in the blood of nephrectomized animals.[1] The following demonstration (Fig. 7) of a close relation between the increased angiotensin yield and the total amount of angiotensinogen in plasma however makes it highly probable, that the change in angiotensin yield is due to the parallel increase in angiotensinogen concentration in plasma. This increase in angiotensinogen content of plasma of nephrectomized animals, which after 24

1 A hypothesis including this view was advanced at the second international congress for nephrology, Prague 1963.

hours is about 10 times the normal concentration, can most simply be explained by lack of a physiological renin release, which normally splits most of the angiotensinogen

The present explanation of the mechanism of the increased sensitivity to renin in nephrectomized animals agrees with results of many previous studies The increased sensitivity to renin of DOCA treated (*Silva & Croxatto* 1952, *Masson et al* 1955, *Gross & Sulser* 1956), unilaterally nephrectomized (*Bing* 1962) and unilaterally clamped rats after removal of the clamped kidney (*Gross* 1958) agrees with the decreased renal renin content in these animals In accordance with this explanation are also experiments by *Gross & Sulser* (1956) and *Blaquier et al* (1962), who showed that while nephrectomized rats *injected with* extracts of normal kidneys react with a normal short response, animals injected with renin-poor extracts of kidneys from DOCA treated rats show the typical prolonged reaction to intravenously injected renin

The increased yield of pepsitensin and anephrotensin in plasma from nephrectomized or DOCA treated animals (*Rosas & Croxatto* 1961) is also in accordance with the idea, that the increased yield of angiotensin is due to a change in substrate, all three peptides having their main or only origin in the angiotensinogens

SUMMARY

In studies on the nature of the humoral factor which potentiates angiotensin formation in plasma of nephrectomized animals, the degree of potentiation was determined as the ratio between the yield in a hog-horse or human-monkey renin angiotensinogen system and the yield in the same system after replacement of an amount of saline with the same amount of plasma This ratio of yields from

$$\frac{\text{renin} + \text{plasma} + \text{angiotensinogen}}{\text{renin} + \text{saline} + \text{angiotensinogen}}$$

which was about 1 in normal rats, was from about 2 to about 4 in nephrectomized rats (*Fig 1*) The same values for normal and nephrectomized guinea-pigs were about 2 to 6 and 5 to 9 respectively (Fig 2)

Further studies (Tables 2 and 3 and Figs 3 and 4) showed similarities between angiotensinogen(s) in normal plasma and the potentiating factor(s) in plasma from nephrectomized animals they have the same species specificity, are non dialysable and found in the supernatant after dialysis, are destroyed by heating to 56° C for half an hour, by pepsin and by renin As further studies (Fig 7) show that the increases in angiotensin yield parallels increases in the angiotensinogen concentration, which 24 hours after nephrectomy is about 10 times the normal value, it is highly probable that the increased sensitivity to renin in these animals is caused by the increase in angiotensinogen content

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FROM THE RENAL WARD IV MEDICAL DEPARTMENT (HEAD PROFESSOR B v BONSDORFF) AND THE DEPARTMENT OF PATHOLOGY (HEAD PROFESSOR E SAXÉN) UNIVERSITY OF HELSINGFORS MARIA HOSPITAL HELSINGFORS FINLAND

RENAL HISTOPATHOLOGY IN A CASE OF NEPHROPATHIA EPIDEMICA MYHRMAN

A Study of Successive Biopsies

By

BÖRJE KUHLBÄCK, PER FORTELIUS and LEIF G TALLGREN

Received 12 vii 63

Nephropathia epidemica (n e), first described by *Myhrman & Zetterholm* in 1934, is an acute renal disease occurring in northern Scandinavia It is believed to be due to a virus Several cases have been described from both Sweden (*Loster & Lublin* 1950, *Myhrman* 1951, 1957, *Floderus* 1957, *Fjelistrom* 1960), Norway (*Knutrud* 1949, *Muri* 1950) and Finland (*Stuhlfauth* 1943, *Hortling* 1946) The onset of the disease is characterized by high fever, chills, headache and pain in the back, abdominal pain oliguria, haematuria and uraemia It is not preceded by streptococcal or staphylococcal infection Oedema and hypertension do not occur The symptoms usually subside rapidly It is sometimes epidemic, and wood mice and voles are believed to be vectors of the virus

Later, it has been found that n e has many features in common with Far Eastern haemorrhagic fever and haemorrhagic nephroso nephritis, which occur in northern Asia and Russia (*Gajdusek* 1953, *Warren* 1955, *Smorodintsev, Chudakov & Churilov* 1959) These diseases, which are regarded as identical and are caused by a virus, are characterized by generalized capillary damage and haemorrhages, chiefly affecting the kidneys Recently it has again been suggested that n e may be a milder form of this Asiatic virus disease (*Ornstein & Soderhjelm* 1963)

Data regarding the pathological anatomy in n e have been lacking A case corresponding in all respects to the criteria of the disease in question was recently treated in the Renal Ward of the IV Medical Department, Maria Hospital, Helsingfors

CASE REPORT

J no 122 62 Male 36 timber worker Nothing noteworthy in regard of heredity As a child the patient was in good health In 1940 he was hospitalized for tetanus He had very seldom been ill There was no history of tonsillitis gastric disorders

This increase in angiotensinogen is believed to be due to an accumulation, caused by lack of a physiological renin release

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per cent On Dec 1, owing to the oliguria and the uraemic condition the patient was remitted by aeroplane to the Renal Ward of the Maria Hospital Helsingfors

On admission the patient was exhausted but the sensorium was clear and he was mentally orientated The temperature and blood pressure were normal There was moderate tenderness over the kidneys the pharynx was normal, there was no nausea, and the headache and stiffness of the neck had abated No oedema was noticeable The blood pressure was 120/80 mmHg Examination of the heart, lungs and abdomen revealed nothing noteworthy A roentgenogram of the thorax was normal The blood picture exhibited nothing pathological The ESR was 47 mm/hr The plasma creatinine was 12 1 mg per cent and rose the next day to 13 6 mg per cent

he urinary output

he urine contained

disappeared The

No bacteria were

detected The serum proteins were normal Moderate acidosis was present Serum potassium and *serum sodium were normal Serum magnesium* was 2 6 mEkv/l Serum phosphorus was 7 4 mg per cent and serum calcium 7 4 mg per cent These electrolyte disturbances rapidly normalized The plasma creatinine dropped in four days from 13 6 mg per cent to 1 33 mg per cent A blood culture yielded no growth The antistreptolysin titre was 32 64 and the antistaphylolysin titre 0 56–0 64 A pharyngeal swab gave growth of α haemolytic streptococci and *Neisseria catarrhalis* The bleeding time and coagulation time were normal A culture for *Leptospira* was negative and the antibody titre to tick borne encephalitis was not elevated Further data and the course of the disease appear in Fig 1

The patient soon recovered During the first few days he was given no special therapy apart from correction of the fluid and electrolyte balance On Dec 2 50 mg of Durabolin were administered On discharge three weeks later the patient was completely cured The creatine clearance and phenolsulphonphthalein pitressin and ammonium chloride tests were normal

At a follow up examination five months later the patient was in good health The urine contained no proteins or blood and the plasma creatinine was normal (0 7 *mg per cent*) All renal function tests were normal Renal angiography showed a normal vascular distribution in the kidneys

During the illness four percutaneous renal biopsies were made

1st renal biopsy (on the 9th day of illness) A specimen about 10 mm in length consisting of cortex showing 5 7 glomeruli and medulla to half its length The most striking feature is the presence of abundant haemorrhages in the medulla (Figs 2, 3) These are mostly fresh, but there are also haemorrhages with obvious signs of haemolysis The medullary haemorrhages measure some hundred microns in diameter The interstitium appears to be oedematous and the collagenous fibres appear to be swollen Marked degenerative changes or desquamation are observable in the tubular epithelium, in particular in the area bordering on the cortex Desquamated epithelial cells, isolated or in small groups, are seen in the distal tubules of the medulla (Fig 4) The basal membranes of the medullary tubules appear to be swollen and broadened, with a *diminished PAS reaction* Both in the medulla and in the cortex small, mostly lymphocytic infiltrations are seen, some of which are obviously connected with rupture of a tubule

The cortical changes (Fig 5) are much less striking, consisting of uneven thickenings of the walls of many arterioles, often adjacent to and in part including the glomerular tuft The thickenings are markedly PAS-positive and acidophilic The basal membranes of the glomerular loops also appear to be in part thickened The medulla, too, exhibits

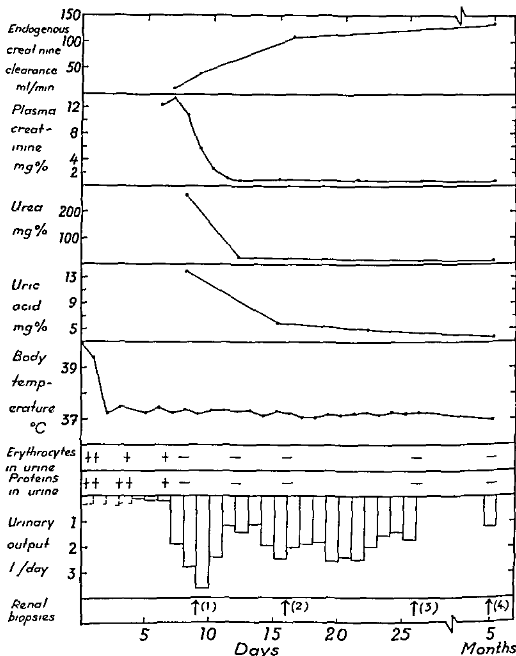


Fig 1
The course of the disease during the stages of oliguria and diuresis

headaches or fever In the autumn of 1962 the patient was doing lumber work in the region of Torneå in the north of Finland until he fell ill

On Nov 25, 1962 there was a sudden onset of high fever (up to 40° C) and diffuse pain in the back The following day the fever was still high and the patient observed that the urine was dark red and scanty in volume In addition he had chills and muscular pains The next day he was admitted to the Torneå General Hospital At this time headache, slight stiffness of the neck and moderate disorientation were also present The temperature dropped but the urinary output was very scanty (about 100 ml/24 hours) Lumbar puncture showed nothing noteworthy There was proteinuria (Esbach 6–20 ‰) The non protein nitrogen in the blood was 90 mg

per cent On Dec 1 owing to the oliguria and the uraemic condition the patient was remitted by aeroplane to the Renal Ward of the Maria Hospital Helsingfors

On admission the patient was exhausted but the sensorium was clear and he was mentally orientated The temperature and blood pressure were normal There was moderate tenderness over the kidneys the pharynx was normal there was no nausea and the headache and stiffness of the neck had abated No oedema was noticeable The blood pressure was 120/80 mmHg Examination of the heart lungs and ab

ax was normal

47 mm/hr The

3 6 mg per cent

urinary output

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detected The serum proteins were normal Moderate acidosis was present Serum potassium and serum sodium were normal Serum magnesium was 2 6 mEkv/l

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titre 0 56–0 64 A

course of the disease appear in Fig 1

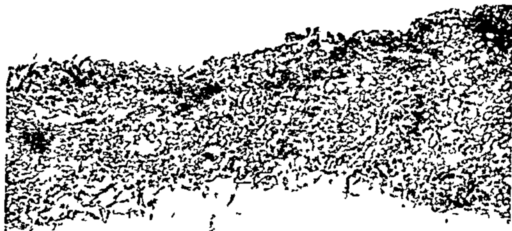
The creatine clearance and phenolsulphonphthalein pitressin and ammonium chloride tests were normal

At a follow up examination five months later the patient was in good health The urine contained no proteins or blood and the plasma creatinine was normal (0 7 mg per cent) All renal function tests were normal Renal angiography showed a normal vascular distribution in the kidneys

During the illness four percutaneous renal biopsies were made

1st renal biopsy (on the 9th day of illness) A specimen about 10 mm in length consisting of cortex showing 5–7 glomeruli and medulla to half its length The most striking feature is the presence of abundant haemorrhages in the medulla (Figs 2, 3) These are mostly fresh, but there are also haemorrhages with obvious signs of haemolysis The medullary haemorrhages measure some hundred microns in diameter The interstitium appears to be oedematous and the collagenous fibres appear to be swollen Marked degenerative changes or desquamation are observable in the tubular epithelium, in particular in the area bordering on the cortex Desquamated epithelial cells, isolated or in small groups are seen in the distal tubules of the medulla (Fig 4) The basal membranes of the medullary tubules appear to be swollen and broadened with a diminished PAS reaction Both in the medulla and in the cortex small mostly lymphocytic infiltrations are seen, some of which are obviously connected with rupture of a tubule

The cortical changes (Fig 5) are much less striking consisting of uneven thickenings of the walls of many arterioles, often adjacent to and in part including the glomerular tuft The thickenings are markedly PAS positive and acidophilic The basal membranes of the glomerular loops also appear to be in part thickened The medulla too exhibits



Figs 2 3

Fig 2 1st biopsy specimen Medulla with haemorrhages corresponding to the dark areas Lillie s allochrome staining Low magnification

Fig 3 1st biopsy specimen Haemorrhage with beginning haemolysis Tubules without any epithelium or with marked degenerative epithelial changes A hyaline cast is observable low down in the middle Lillie s allochrome staining Objektive × 25

vascular changes resembling those seen in the cortex but no obvious ruptures are observed

The picture is suggestive of haemorrhagic nephroso nephritis

2nd renal biopsy (on the 16th day of illness) The specimen measures about 5 mm and consists half of medulla half of cortex with three



Figs 4–5

Fig 4 1st biopsy specimen Area on the border between cortex and medulla Degenerative epithelial changes and desquamation of the epithelium in the tubules some of which are distended Lillie s allochrome staining Objective × 10

Fig 5 1st biopsy specimen Thickenings of the basal membrane in Bowman s capsule and in capillary loops near the vascular pole to the left The afferent arteriole is practically obliterated, showing a thickening on the wall with indistinct borders Scanty inflammatory cells in the interstitium No obvious changes in the tubules Lillie s allochrome staining Objective × 25

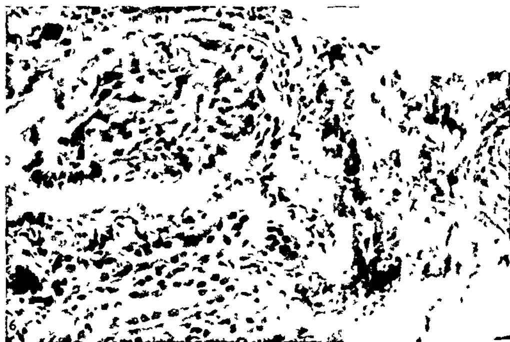


Fig 6

2nd biopsy specimen To the right of a glomerulus a portion of an arteriole is seen in longitudinal section Note the uneven PAS positive staining in the somewhat thickened wall Fine threaded loose fibrosis with inflammatory cells in small numbers, is observable Some tubules are markedly distended Lillie's allochrome staining Objecktive × 25

glomeruli The observable changes are slight The medullary tubules are somewhat irregular and of varying sizes There appears to be an increase in the amount of connective tissue, and the latter exhibits numerous small lymphocytic infiltrations The same holds good in regard of the cortex

The medulla exhibits tubules with incomplete epithelium and occasional detached fragments of epithelium with degenerative changes Collapsed tubules surrounded by inflamed areas are observable Many tubules in the medulla have a basophilic epithelium with large nuclei indicative of regeneration

Both in the cortex and in the medulla there are small blood vessels with walls showing PAS positive, often nodular unevenly staining thickenings (Fig 6) These changes appear to be slighter than in the previous specimen

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Figs 7 8

Fig 7 3rd biopsy specimen Two glomeruli and tubules exhibiting no definite changes Beneath the glomerulus to the left an arteriole with a clearly distinguishable PAS positive thickening Lillie's allochrome staining Objective × 25

Fig 8 3rd biopsy specimen A glomerulus with obvious signs of necrobiosis in a lobe which appears to be herniated into a proximal tubule Lillie's allochrome staining Objective × 25



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Fig 8 3rd biopsy specimen A glomerulus with obvious signs of necrobiosis in a lobe which appears to be herniated into a proximal tubule Lillie's allochrome staining Objective × 25

On evaluating this finding incipient arteriolo-hyalinosis was regarded as a possible alternative However, the nodular character of the thickenings the indistinct border and the picture of regression yielded by the later biopsy specimens were indicative of an acute change bearing a causal relationship to the clinical picture

Comparison with the renal changes described by *Smorodintsev et al* (1959) in an autopsy material comprising cases of Asiatic haemorrhagic nephroso nephritis revealed a striking resemblance with the histological findings in the present case In particular, attention was drawn to the changes in the small blood vessels, described by these authors as generalized and regarded by them as pathognomonic of haemorrhagic nephroso nephritis These vascular changes were obviously identical with those observed in our histological material

Medullary haemorrhages, signs of severe tubular damage, interstitial oedema and scanty inflammatory infiltrations were also described by *Smorodintsev et al*, and by *Oliver & MacDowell* in epidemic haemorrhagic fever Haemorrhages were only observed in our first biopsy specimen which in addition showed beginning haemolysis (Fig 3), constituting evidence against the possibility of the biopsy procedure being the cause of the haemorrhages The fact that fresh haemorrhages were still observed on the 9th day of illness is in agreement with the observations made by the above-mentioned authors According to them, oedema is typical during the first few days, while haemorrhages occur somewhat later

As far as can be judged on the basis of the biopsy specimens, the tissue reactions following the acute changes were slight, being thus in accordance with the clinical course of the disease The isolated necrosis (Fig 8) observed in one glomerulus also corresponds to the changes reported by *Smorodintsev et al* and is obviously not necessarily of any major clinical importance (cf Fig 6 showing glomeruli which are normal in appearance)

Radosevic & Mohacek (1954), who described some cases of n e from Jugoslavia regarded the disease as an acute interstitial nephritis, resembling *that seen after* sepsis, certain intoxications blood transfusion reactions etc They thus regarded the disease as identical with acute renal failure or lower nephron nephrosis Unfortunately, these authors were not able to *confirm* their assumption by any histological data In our opinion, there is a certain resemblance between the histological picture in n e and acute *tubular* necrosis, in particular as regards the tubular changes but the vascular changes the type of the haemorrhages and the glomerular changes are features differing markedly from those typical of acute tubular necrosis In acute glomerulonephritis however such a picture may occasionally occur

Cell and virus cultures *of the* first biopsy specimen yielded no data regarding the aetiology of the disease in the present case Probably the

time that had elapsed since the onset of the illness (9 days) was too long for a virus culture to succeed

In our case the benign course of the disease was very striking The histological changes appeared reversible In spite of severe oliguria and uraemia the patient recovered in a surprisingly short time, and there was no need for haemodialysis In this respect the disease seems to differ from the Asiatic haemorrhagic nephroso nephritis, in which a much more grave, and sometimes lethal course has been reported In contrast to acute glomerulonephritis, which often causes severe renal lesions and may lead to chronic nephritis, n e appears to cause no persistent renal damage The return to completely normal renal function is in good agreement with the histological finding in the present case

SUMMARY

The course in a typical case of nephropathia epidemica is decribed The onset of illness was sudden, with high fever, pain in the back and headache, abdominal pain and disorientation At the same time there were signs of severe renal damage, with oliguria, proteinuria, haematuria and uraemia The symptoms rapidly subsided Four percutaneous renal biopsies were made at different stages of the disease The most striking histological findings consisted of medullary haemorrhages nodular thickenings on the walls of arterioles and glomeruli, signs of severe tubular damage and interstitial oedema The fourth biopsy specimen, obtained five months after the beginning of the disease, was histologically normal The patho-anatomical picture is discussed, and its resemblance to that of Asiatic haemorrhagic nephroso-nephritis and epidemic haemorrhagic fever is emphasized

ADDENDUM

During the preparation of this paper another case has occurred showing some of the features typical of nephropathia epidemica On anamnestic grounds however this case rather belongs to the above mentioned group of Far Eastern haemorrhagic fever and haemorrhagic nephroso nephritis Thus this case may constitute a missing link between these and the Scandinavian disease entity

A 24 year old nurse suddenly fell ill in the city of Tammerfors Finland on the 6th of January 1963 The symptoms consisted of fever abdominal pain oliguria

the appendix was

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discharged a few

weeks later A percutaneous biopsy specimen obtained on the fifth day of illness exhibited a histological picture similar to that seen in the case described in this paper, i e the peculiar nodular changes in the walls of some arterioles medullary haemorrhages focal degenerative changes of the medullary tubules medullary and cortical oedema and thickening of the glomerular basal membran in some glomeruli

It is of interest that the patient s brother in law visited her family 40 days prior to her illness He had just arrived from Russia where he had been engaged in work in Siberia He was taken ill with acute reversible uraemia 25 days after his arrival Furthermore the patient s brother who also had stayed with their Siberian guest

fell ill 19 days later than his sister developing acute serious uraemia The patient s brother in law as well as her brother recovered rapidly without specific nephro log cal therapy Data regarding the course of the latter cases are lacking however

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The uncertainty which prevails in respect to the aetiology of these diseases their interrelationship and classification, is distinctly evident from the variety in the nomenclature Most recently, *Lichtenstein* (6), has collected them under the designation histiocytosis X owing to the underlying histiocytic proliferation and the unknown aetiology In the same way, the Lancet has suggested the common term Letterer Christian disease in an attempt to do away with the isolated terms E G, H S C, and L-S whose definitions are getting ever more arbitrary as one transitional case after the other is reported

The gradually very extensive literature on this subject has been reviewed several times, and we shall emphasize only a few main features E G was first described in 1935 by *Fraser* who distinguished this special histological appearance from the so called xanthomatoses collected as Hand Schüller Christian s disease He reported four cases of lipid granulomatosis with eosinophilia All these patients had multiple bone lesions and one had diabetes insipidus The histological appearances corresponded to those which have later been described as E G The term eosinophilic granuloma was used for the first time by *Lichtenstein & Jaffe* (7) in 1940 They described two cases with solitary skeletal destructions containing densely packed macrophages with strands of eosinophilic granulocytes In the same year *Otani & Ehrlich* (8) described four similar cases which they called solitary granuloma of bone *Jaffe & Lichtenstein* (4), as well as *Engelbreth Holm, Teilum* and *E Christensen* (2) in 1944, suggested for the first time that E G, H S C, and L-S might be different clinical manifestations of the same basic disease affecting with predilection haemopoietic tissue In these authors' opinion it presumably repr... inflammatory reaction to an

Accordi... marily characterized by the presence of a large number of histiocytes arranged in strands interspersed by groups of eosinophilic cells, mainly eosinophilic granulocytes Furthermore, giant cells are present often in the margin of necrotic areas In relation to the necroses there are frequently sudanophilic vacuoles in the histiocytes

This basic lesion may according to *Jaffe & Lichtenstein* (6), manifest itself in three ways

..., solitary or multiple)
(H S C)
subacute (L-S)

In other words a continuous spectrum from a solitary, non progressive skeletal lesion through multiple, more or less progressive skeletal lesions to an acute disseminated condition in which the skeletal lesions do not predominate, although they are rarely absent

At a time while the conflict between the two views has not yet been settled it is of some importance to publish cases which are difficult to

DEPARTMENT OF PATHOLOGY (HEAD K. SCHOURUP, M.D.)
THE GLOSTRUP HOSPITAL, DENMARK

EOSINOPHILIC GRANULOMA WITH TRANSITION TO RETICULUM CELL SARCOMA AND EOSINOPHILIC GRANULOMA OF AN ATYPICAL PRIMARY SITE

By

P. CHRISTOFFERSEN and A. RICHTER NIELSEN

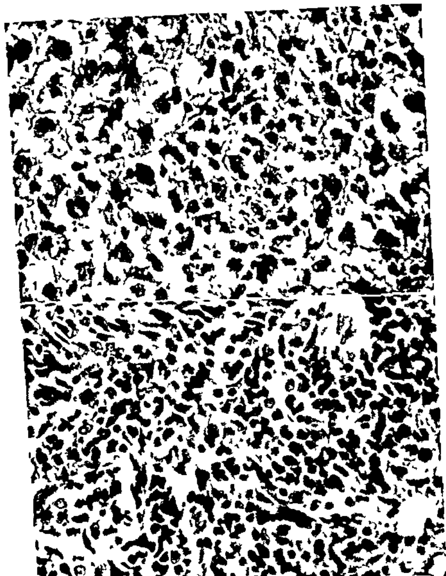
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Despite numerous case reports, major analyses, and detailed histological and biochemical studies, eosinophilic granuloma (E. G.), Hand-Schuller-Christian's disease (H-S-C), and Letterer-Siwe's disease (L-S) remain a mystery. This applies not only to their aetiology and nosology, but also to their interrelationship as well as their relation to other diseases of the reticuloendothelial system.

To-day it is a generally accepted notion that the primary change in these diseases is proliferation of the reticulum cells of the reticular connective tissue, especially the fixed macrophages. The diseases manifest themselves primarily in tissues built up of a reticular stroma, such as bone marrow and lymph nodes, but also in tissues which primarily lack a reticular stroma, *e.g.* the skin. In these latter tissues, the reticulosis results from a differentiation and proliferation of the primitive reticulum cell in the perivascular connective tissue.

This proliferation takes place as a reaction to an unknown, perhaps infectious agent. It is conceded, moreover, that these diseases have no bearing on the so called lipid reticuloendothelioses, such as Gaucher's disease or Niemann-Pick's disease. Their primary factor is an abnormal lipid metabolism with a consequent deposition of lipid in the cells of the reticuloendothelial system, while the cholesterol-containing foam cells seen in E. G. and mainly in H-S-C, arise by phagocytosis of necrotic tissue. It is a characteristic, among others, that these foam cells are not encountered until some months after the onset of the disease. Therefore, these three diseases have been designated as non lipid reticuloendothelioses.

At present, the question is whether we are dealing with different manifestations of the same basic disease, or with three different disease entities. *Siwe & Olani* (9) maintain that combining these three diseases is an over-simplification and that they represent three different diseases which may merely be identical morphologically. In contrast, *Jaffe & Lichtenstein* (4) believe that the three syndromes are variations of the same basic lesion.



Figs 1 2

Fig 1 Densely packed pale reticulum cells with distinct cell borders an l oval o kidney shaped nuclei varying in stal ility × 360

Fig 2 Cells f varying appearances predominated by small cells with a dark pc or ly del mited cyt plasm A s ngle m t sis is seen × 360

pale and vacuolated cytoplasm distinctly delimited, and some smalle cells presenting a dark poorly delimited cytoplasm The appearance were predominated by cells of the latter type (Fig 2) In addition the was a diffuse admixture of lymphocyte like cells with round dar

classify in order thereby to fill parts of the gaps in our knowledge regarding the clinical features and pathology of these diseases We are, therefore, presenting two cases which initially gave rise to diagnostic problems

CASE REPORT

Case 1

A 35 year-old woman who had never been seriously ill was admitted after shooting pain in the right shoulder had persisted for the past three months X rays revealed a large destructive process involving the neck of the humerus and measuring 9 × 4 cm In removing a biopsy specimen the surgeon worked his way through normal periosteum into a cavity containing greyish soft and friable tissue Histological diagnosis Eosinophilic granuloma She was then treated by local X ray therapy which had only a slight and transient effect Three months later another course of X rays because of a recurrence Six months after the first treatment the patient was re admitted because of increasing swelling of the soft tissues around the right shoulder Now, biopsy showed reticulum cell sarcoma, and interthoracoscapular amputation was performed

Owing to constant phantom pain the sensory branches to the brachial plexus were later cut

At a follow up, one year after the amputation, no signs of recurrence were demonstrable

The E S R was 5 when the patient was first seen 27 at the time of the amputation, and 12 at the follow up one year later The eosinophilic granulocyte count was 606/µl when she was first seen

Microscopic Findings

First biopsy Small flakes of tissue predominated by large, polygonal and densely packed cells with an oval to kidney-shaped nucleus varying in stainability from leptochromatic with one or two distinct nucleoli to highly hyperchromatic without nucleoli These cells had distinct cell borders, and the ample cytoplasm was pale and vacuolized (Fig 1) From these cells there were all transitions to smaller cells having a less well-defined darker cytoplasm In certain areas these latter cells appeared to form syncytial patterns There was an increased number of normal mitoses in these cells Furthermore, there were scattered giant cells with 3–4 nuclei and lastly a considerable number of eosinophilic granulocytes which were partly scattered diffusely but particularly arranged in strands through the tissue Wilder staining for reticular fibres showed in certain areas a delicate reticulum network surrounding the individual cells, while other areas were entirely devoid of reticular fibres The reticular fibres were present in the areas which were predominated by the smaller, darker cells In the same areas there were only a few eosinophilic granulocytes, only scattered vacuolized cells, but mitoses were more abundant than in the other areas

Histological diagnosis Eosinophilic granuloma

Second biopsy and operative specimen The tissue consisted of densely packed cells, apparently without a connective-tissue stroma The nuclei were round to oval, and notched in places The chromatin pattern ranged from clear, vesicular, to completely pyknotic The quantity and stainability of the cytoplasm varied also, some cells having an ample,

This latter tumour tissue is indubitably malignant, of the type reticulum cell sarcoma The same elements were present in small quantities in the first biopsy in which there were all transitions from elements typical of E G to malignant reticulum cells

Case 2

A three year old girl Three weeks prior to admission she had recovered from whooping cough During this disease she had developed a small nodule on the right side of the back of her neck This nodule had been growing and was tender Another two nodules have appeared in the same region During the week before admission she had been very tired and had little appetite On admission she was afebrile and the only objective findings were slightly enlarged tender lymph nodes on both sides of the neck and two pea sized firm masses in the nuchal region which were movable against the underlying tissue but adherent to the skin

The two masses were removed They were soft and lobulated One extended right to the pericranium while the other one was of a more superficial localization

Histological diagnosis Acute lymphadenitis with eosinophilia

Three months later the patient was re admitted because of an increasing limp on the left No history of trauma X rays of the pelvis revealed an irregular articular surface in the left acetabulum surrounded by mild halisteresis and a few irregular translucencies in the marginal areas of the acetabulum X ray diagnosis Left sided coxitis

There was no recurrence in the nuchal region, but hazelnut sized lymph nodes in the left axilla and a number of lymph nodes of a similar size in both groins The nodes were non tender A new biopsy was removed from an inguinal node which showed the same histological appearance as on the first occasion A biopsy specimen of the bone marrow from the iliac crest revealed hyperplastic myelopoiesis with a slight shift to the left but without eosinophilia Two weeks later the X ray examination of the pelvis and hip joints was repeated Now there was a cystic translucency in the lower part of the ilium X rays of the skull and other bones showed normal appearances

Operation was decided upon Through an approach along the inner side of the wing of the ilium the inner cortical layer was chiselled off, and at the linea terminalis a cavity filled with soft gelat[illegible] yellow [illegible] ed speci[illegible] without [illegible]

Follow up X rays two months after the operation showed distinct healing At follow up one year after the operation the general condition was good There were still a few enlarged and non tender lymph nodes Gait completely normal

Microscopic examination

Biopsy 1 Small rather firm lymph nodes whose cut surface showed greyish moist tissue with scattered yellowish foci

The lymphatic tissue showed severe transformation, several major and minor abscesses being demonstrable or necrotic areas with neutro

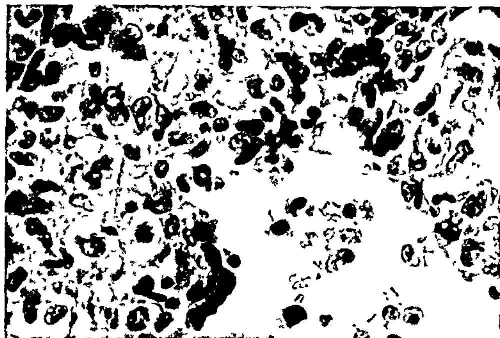


Fig 3
Transitions between the cells seen in Fig 2 × 360

nuclei, surrounded by a narrow rim of cytoplasm Moreover, all transitions between the large cells with a vacuolized pale cytoplasm, via the smaller, darker cells, to the small, lymphocyte-like cells (Fig 3) were noticed There was a slight, diffuse admixture of eosinophilic granulocytes and small areas showing neutrophilic granulocytes Only minor areas gave a positive reaction for reticular fibres, the fibres surrounding isolated cells or small groups of cells

Histological diagnosis Reticulum cell sarcoma

The relationship between E G, H-S-C, and L-S on the one hand and the true reticuloses or reticulohistiocytoses on the other, is far from having been elucidated Several authors maintain that the acute type, L-S, is in fact identical with reticulum cell sarcoma *Bjerre Hansen* (3) has reported a case, a 4-year old boy with co-existing E G and reticulum cell sarcoma of separate origin Perusal of the literature did not disclose any cases of E G with transition to reticulum cell sarcoma On the other hand, there have been several examples of misinterpretation of biopsies from such non-lipid reticuloendothelioses as reticulosarcoma, Hodgkin's disease, etc

In our case the disease started as E G The biopsy specimen was predominated by tissue of a structure like typical L G In addition to these predominant areas, there were smaller areas built up of smaller, darker cells with a sparse, non vacuolized cytoplasm

The operative specimen was predominated by these small dark cells with abundant mitoses However there were still isolated or grouped, large and pale vacuolized cells and areas showing typical eosinophilic

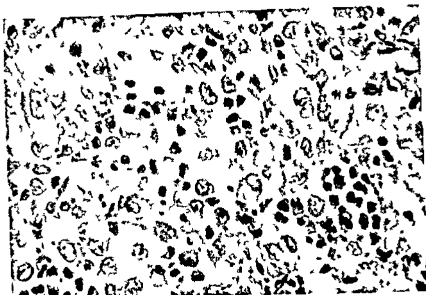


Fig 5
Reticulum cells and eosinophilic granulocytes × 360

ration mainly as strands through the tissue (Fig 4) The capsule was well preserved in most sites but in places reticulum cells and eosinophilic granulocytes were continuing through the capsule in a periglandular infiltration consisting of vacuolized reticulum cells and eosinophilic granulocytes Staining for reticular fibres showed a content normal for lymphatic tissue No bacteria or fungi

Histological diagnosis Acute lymphadenitis with eosinophilia

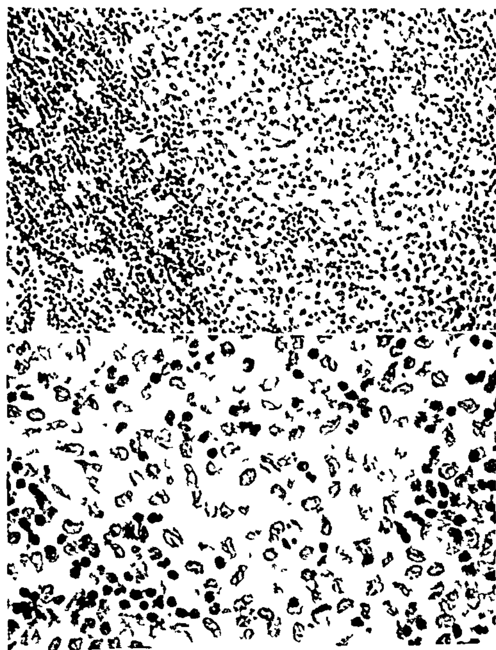
Biopsy 2 showed the same gross and microscopic appearances apart from the absence of periadenitis

Tissue from the bone cyst

A very scanty stroma with a few thin walled vessels The tissue consisted of polygonal densely packed cells with a pale and vacuolized cytoplasm Interspersed between these cells were wide strands consisting of eosinophilic granulocytes (Fig 5) Fat staining revealed small sudanophilic droplets in the pale vacuolized cells The cells were regular with uniform leptochromatic nuclei and a few typical mitoses Small areas were characterized by regeneration and contained newly formed osteoid tissue with osteoblastic activity

Histological diagnosis Eosinophilic granuloma

One of the factors which is emphasized by the opponents of the unitarian theory is the lacking clinical transition between the three syndromes E G H S C and L S *Engelbreth Holm Teilum & E Christensen* (2) 1944) however had 5 cases in which there was clinical transition from E G to H S C



Figs 4 4a

Fig 4 Pale reticulum cells together with lymphocytes and numerous eosinophilic granulocytes × 144

Fig 4a Higher magnification of Fig. 4 × 360

philic granulocytes and nuclear remnants. These foci were surrounded by a rim of varying width made up of large polygonal densely packed pale and partially vacuolized reticulum cells. There was a less marked reticulum cell proliferation throughout the lymphatic tissue. Moreover scattered giant cells with 2-4 nuclei were seen, and finally a considerable number of eosinophilic granulocytes partly diffuse in the area of suppu-

female mouse Each animal weighed about 25 g Each mouse received 10^{6} cells from the spleen and the lymph glands by the transplantation

A total of 11 animals received treatment with streptococcal lysate, 11 animals received placebo, and five animals remained untreated One animal died following the first placebo injection, while the remaining animals all died of leukaemia between the 13th and 29th day after the transplantation The treatment had no effect on the survival time (Table 1) The total dose per g body weight varied from about one to eight times the optimal dose of lysate administered to young rabbits with Brown-Pearce carcinoma (2)

TABLE 1

AKR MICE with Leukaemia Treated with Streptococcal Lysate The Median Survival Times Are Tabulated as the Median with a 95 per cent Confidence Interval (5)

Treatment per day	No of animals	Median survival time in days
2 or 3 ml of lysate	6	26.5 ± 6.0
2 or 3 ml of placebo	5*	16.0 ± 6.5
0.5 or 1 ml of lysate	5	22.0 ± 5.0
0.5 or 1 ml of placebo	5	26.0 ± 2.5
Untreated	5	25.0 ± 2.0

* One animal died after the first placebo injection

The *second series of experiments* consisted of a total of 30 *dba 212* m... 15 ...

leukaemia)

TABLE 2

Dba 212 Mice with Ascites Tumour (Lymphatic Leukaemia) Treated with 0.6 ml Streptococcal Extract per Day for five Days The Number of Free Cells in the Peritoneum is Tabulated as the Median Value with a 95 per cent Confidence interval (5)

Treatment	No of animals	Sex	No of free cells in the peritoneum
Extract	10	♂	$2.6 \pm 0.44 \times 10^{8}$
Placebo	5	♂	$3.2 \pm 1.51 \times 10^{8}$
Extract	10	♀	$3.4 \pm 0.83 \times 10^{8}$
Placebo	5	♀	$3.2 \pm 0.93 \times 10^{8}$

A total of 10 male and 10 female mice received treatment with streptococcal extract, while 5 male and 5 female mice received placebo All the animals were ...
the number
ment had no

case of lymphatic leukaemia With the same motives, the total number of cells in ascites fluid from *dba 212* mice with a transplantable ascites tumour (lymphatic leukaemia) was examined to see whether this could be influenced, and finally, Ehrlich ascites carcinoma cells were studied to see whether they could be inhibited *in vitro*

MATERIAL AND METHODS

Both AKR mice and *dba 212* mice were used in the experiments, these two mouse strains have been maintained for many years at the University Institute of Pathological Anatomy, Copenhagen

The tumours used were partly first generation transplants of a spontaneous lymphatic leukaemia in an AKR mouse and partly a transplantable ascites tumour which had originated as lymphatic leukaemia in a *dba 212* mouse in December 1961 and maintained since then as ascites tumour in *dba* mice through about 60 transfers at the Fibiger Laboratory, Copenhagen Ehrlich Landschutz hyperdiploid ascites carcinoma was used for the *in vitro* experiments

Transplantation to the AKR mice was performed by intravenous injection of 10 cells from the spleen and lymph glands of an AKR female mouse with spontaneous leukaemia Transplantation to the *dba mice* was carried out by intraperitoneal injection of 5×10^6 cells from a mixture of ascites tumour cells from three donors

In the experiments with AKR mice the effect of treatment was evaluated on the basis of the survival time In the experiments with *dba* mice the total number of free cells in the peritoneum was the basis for evaluating the effect All the *dba* mice were sacrificed seven days after the transplantation the exudate in the peritoneal cavity was aspirated the peritoneum was washed out with physiological saline and the ascites fluid plus washings were mixed and diluted to a total of 10 ml The total number of free cells in the peritoneum was then determined by counting in a Burker Turk haemocytometer so that the amount of ascites fluid per animal was not involved in the evaluation of the results The values obtained were evaluated by the method indicated by *Dean & Dixon* (5)

The Ehrlich Landschutz hyperdiploid ascites carcinoma was cultured as a monocellular layer in Eagle s medium with 10 per cent calf serum Each flask contained 4 ml of substrate The day after the cultures were established the initial number of cells per flask was determined by counting the cells in 10 flasks At the daily exchange of medium 60 or 120 µl of streptococcal extract was added to each flask The cell count at the beginning and end of the period of cultivation was done in a Bürker-Turk haemocytometer and the results were evaluated according to the procedure indicated by *Dean & Dixon* (5)

In the experiments with AKR mice the animals received treatment with a lysate of *Streptococcus pyogenes* Group A Type 12 3465 (local isolation number) prepared by lysing the culture with sewage phage (4) The treatment was performed by daily intraperitoneal injections of from 0 5 to 3 0 ml from the day following the transplantation Human serum broth (9) was used as placebo

In the experiments with *dba 212* mice the animals received treatment with an extract of the streptococcal strain mentioned above The extract was prepared by grinding the bacteria with alumina and extracting with isotonic phosphate buffer saline pH 7 38 (3) Treatment consisted of daily intraperitoneal injections of 0 6 ml starting two days after the transplantation Isotonic phosphate buffer saline was used as placebo

The lysate and extract used in the above mentioned experiments had been shown to have an inhibitory effect on Brown Pearce carcinoma in experiments on young rabbits

RESULTS

The *first series of experiments* comprised a total of 27 male AKR mice, constituting the first transfer (first generation transplants) of a lymphatic leukaemia which had arisen spontaneously in an AKR

mice, as a sensitive but at the same time simple assay system is necessary if the investigations into the tumour-inhibiting effect of streptococci are to be continued effectively

SUMMARY

Preparations of haemolytic streptococci with an inhibitory effect on Brown Pearce carcinoma in young rabbits were examined for an inhibitory effect on

1 Leukaemia in first transfer (first generation transplants) in AKR mice

2 Ascites tumour in *dba 212* mice (lymphatic leukaemia in the form of ascites tumour in 60th transfer)

3 Ehrlich Landschutz hyperdiploid ascites carcinoma in *vitro*

Even though the dose per g animal weight was up to five times as great as the smallest dose effective on Brown-Pearce carcinoma, no inhibition of the mouse tumours could be demonstrated

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3

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extract used per g weight of animal was about five times as great as the smallest effective dose in experiments with Brown-Pearce carcinoma in young rabbits (3).

In the *third series of experiments*, Ehrlich-Landschutz hyperdiploid ascites carcinoma cells were subjected to treatment *in vitro* with extract of streptococci in concentrations which either corresponded to the doses used for treatment of the animals in the second series of experiments, or to the half of these doses. As indicated in Table 3, the treatment had no effect on the cells.

TABLE 3

Ehrlich-Landschütz Hyperdiploid Ascites Carcinoma Cells Treated in vitro with Streptococcal Extract. The Number of Cells Is Tabulated as the Median Value with a 95 per cent Confidence Interval (5)

Treatment	No. of flasks	No. of cells per flask at beginning of the experiment	No. of cells per flask at the end of the experiment
No treatment	10	$1.0 \pm 0.14 \times 10^6$	
60 µl of extract per 4 ml substrate	10		$6.0 \pm 1.10 \times 10$
No treatment	9		$7.3 \pm 1.45 \times 10^6$
No treatment	10	$1.7 \pm 0.20 \times 10^6$	
120 µl of extract per 4 ml substrate	10		$5.7 \pm 0.77 \times 10$
No treatment	10		$6.6 \pm 1.45 \times 10$

DISCUSSION

It is well-known that the toxicity of tumour-inhibiting substances can vary considerably from one animal species to another. Similarly, different tumours in the same species can have very varying sensitivity to the same substance. For example, Shear's polysaccharide (13), prepared from Serratia marcescens, varies greatly in its toxicity for different animal species (1). Mice can tolerate far greater doses than guinea pigs, dogs, rabbits and man, and the tumour inhibition which it has been possible to demonstrate in experiments with a variety of mouse tumours has not been achieved either in rabbits (14) or in man (12). In 1961, *Havas et al.* reported that mixed preparations from haemolytic streptococci and S. marcescens could inhibit sarcoma 37 and Krebs-2 carcinoma in mice, while no effect was found on six other tumours, *viz.* three transplantable mouse tumours, spontaneous mammary carcinomas and methylcholanthrene induced tumours in mice and *on Rous sarcoma* in chickens (6). On the basis of this knowledge it was not surprising to find that the streptococcal preparations used in the present experiments proved to be a further example of difference in tumour-inhibiting effect on different tumours.

It is intended to continue the experiments with other tumours in

FROM THE RHEUMATIC RESEARCH LABORATORY, UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY, COPENHAGEN (PROFESSOR G. TEILUM, M.D.)

ELECTRON MICROSCOPICAL STUDIES OF GRANULATION TISSUE FORMATION OF ANIMALS TREATED WITH ANTIRHEUMATIC COMPOUNDS

By

OLE JØRGENSEN

Received 16.vii.63

it was proposed that the
inl ad healing might be due
to wn that treatment with
glucocorticoid hormones, phenylbutazone, and sodium salicylate resulted in a reduction of the relative water content of granulation tissue of open wounds without changes of the collagen concentration. Treatment with all 3 compounds resulted in morphological changes af the individual fibroblasts accompanied by loss of orientation of the cells and reduced amounts of intercellular substance. The purpose of the present paper is to investigate and compare possible changes of the ultrastructure of granulation tissue of open wounds due to treatment with prednisone, phenylbutazone, and sodium salicylate.

MATERIAL AND METHODS

Fem
200
sub
as d days after wounding granulation tissue of control animals was removed. Granulation tissue of treated animals was removed 8 days after wounding.
Th

RESULTS

4 days after wounding cells of extremely varied morphology are seen scattered in the tissue. Some of these cells contain very small amounts

This investigation was aided by a grant from the *Danish National Association against Rheumatic Diseases*. Part of the investigation was carried out in the Laboratory for Investigation of the Pharmacology of the Connective Tissue at the Department of Pharmacology.

The author is thankful to *F. Carlsen, M. Sc., University Institute of Biophysics* for kind permission to use the electron microscope of the institute.

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Fig 2

Normal fibroblast, 4 days old The e r consists of saccular formations, not very rich in granules Granular bodies and young mitochondria are seen Note extensive Golgi zone Magnification 46500



Fig. 1

Fibroblast of normal group, 4 days old Mitochondria at several stages of development are seen Relative little e r with very few granules In the extracellular space a dark mass of fine fibrils is seen Magnification 45500

of endoplasmic reticulum (e r) and few other organelles These cells probably represent undifferentiated mesenchymal cells In others higher differentiation of the cytoplasm is seen The e.r is, however, in most cases rather sparse consisting of short saccular formations or rounded profiles with small numbers of granules unevenly distributed on the outer side (Figs. 1–2). In the extracellular space accumulations of unbanded fibrils (Fig 1) and a few collagen fibrils are seen Eight days



Fig 4

Parts of several fibrol lasts of normal group (8 days old) Note distinct cell borders In the extracellular space fine collagen fibrils Magnification 29000

Fig 3

Part f cytoplasm of 8 days old fibroblast of normal group Well developed e r with many granules Dilated mitoch ndria Magnification 40500

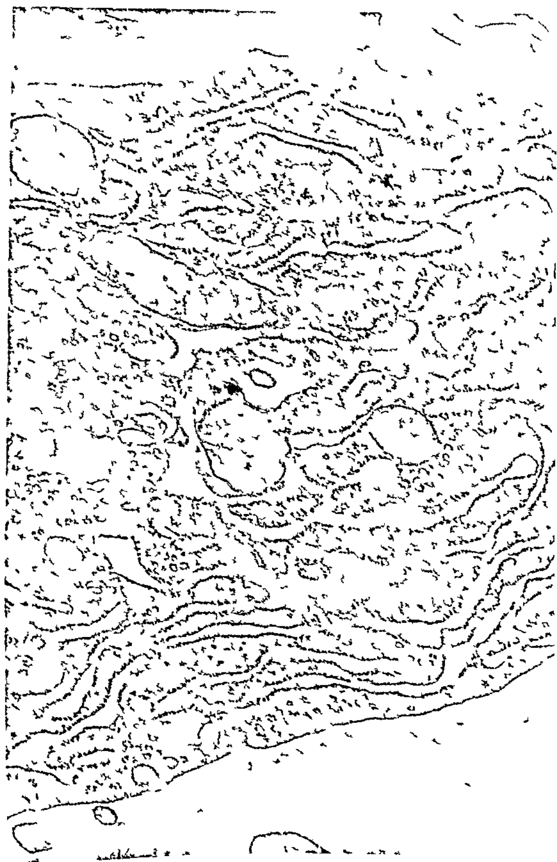


Fig. 3

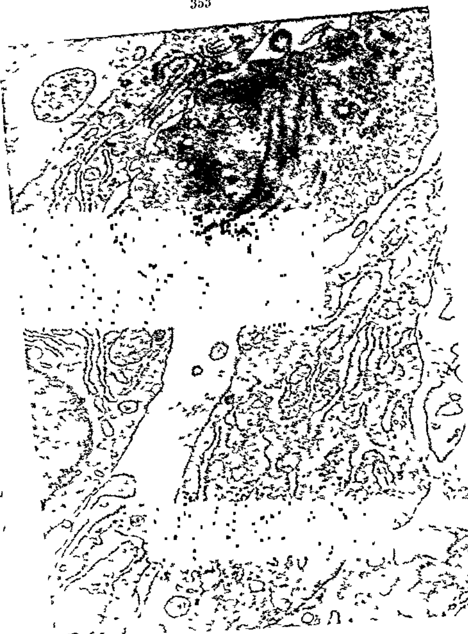


Fig 4
Parts of several fibr blasts of normal group (8 days old) Note distinct cell bord
In the extracellular space fine collagen fibrils Magnification 29000

Fig 3
Part of cytoplasm of 8 days old fibroblast of normal group Well developed
with many granules Dilated mitochondria Magnification 40500



F g 5

1⁹ days ol l f brol last of normal gr ip Only little e r is seen with rather few gra nules A fine fil rillar material can l c seen in the c toplasm M gnif cat on 30000

after wounding the morphology of the fibroblasts appears more uni form the greater part of the cells containing a well developed e r with great amounts of granules placed with regular intervals on the outer side In these cells the e r fills the greater part of the cytoplasm In the

cytoplasm small amounts of ribosomes and some microvesicles are seen (Figs 3–4) In the extracellular space increasing amounts of fine collagen fibrils are found, often encircling individual cells The dark masses of unbanded fibrils encountered in younger granulation tissues have disappeared completely After 12 days the e r is again decreasing in size with rather few granules often appearing as cystic formations Accumulations of fine granules may be seen in the cytoplasm outside the e r (Figs 5–6) In the extracellular space increasing amounts of collagenous fibrils are seen No condensations of fibrillar material have been found adjacent to the cell membranes in the extracellular space

8 days after wounding the fibroblasts of the prednisone treated group contain less e r than normal cells of the same age The e r bears reduced numbers of RNA granules but in many cells great parts of the cytoplasm are filled with fine granules (Figs 7–8)

Most of the fibroblasts of 8 days old granulation tissue treated with phenylbutazone or salicylate exhibit varying degrees of destruction with expulsion of cellular contents (Figs 9 10) Even in intact cells, however, great changes of the ultrastructure are seen Great variations exist, thus the e r may consist of dilated cisternae filling the greater part of the cytoplasm whereas in other cells little e r is seen Generally, the amounts of RNA granules on the e r are reduced, many granules being found in the cytoplasm In the cells of this group ample amounts of fine fibrils may be seen in the cytoplasm this being a rare finding in normal cells

The fibroblasts of young granulation tissue contain great amounts of granular bodies many of which are surrounded by double membranes In some of these [illegible]

d [illegible]

I [illegible]

I [illegible] granules (Figs 1–2) In later stages, however, the mitochondria dilate with a concomitant disappearance of the granules (Figs 3–[illegible]

of [illegible]

cel [illegible]

Du [illegible] phenylbutazone or salicylate great dilatation of the mitochondria takes place, often accompanied by extreme shortening or even disappearance of the cristae In some cases even destruction of the mitochondria may occur (Figs 9 10) These morphological changes of the [illegible]

of increased [illegible]

strange nuc [illegible]

may be s [illegible] accumulation of mitochondria around



Fig 6

Part of long slender fibroblast of normal group—12 days old Note presence of fibrillar material in apparent cystic formations inside the cell These structures probably represent extensions of the extracellular space Very little e r is seen Note granular bodies in apparent dissolution In the extracellular space great amounts of collagen fibrils are seen Magnification 35000



F g 7
S al n d g a ng f l bla s of j dn one t ea d an mal 8 days aft r wound ng N e na sl ts f e w en the ell relat e s a of e r and rath r granula m o hondr a Magn f cat on 45 00



Fig 6

Part of long slender fibroblast of normal group—12 days old Note presence of fibrillar material in apparent cystic formations inside the cell These structures probably represent extensions of the extracellular space Very little e r is seen Note granular bodies in apparent dissolution In the extracellular space great amounts of collagen fibrils are seen Magnification 35000



Fig 9
Fibroblasts in partial disintegration due to treatment with phenylbutazone Many greatly dilated mitochondria are seen some of them in obvious dissolution Note partial destruction of nucleus Magnification 26500

Fig 8
Fibroblasts of prednisone treated group Only little e r and many free granules in the cytoplasm The mitochondria appear rather granular Magnification 49000



I g 8



Fig 11

Detail fr m fibr blast treated with phenylbutazone A gr up of granular b dies and mit chondria in partial resoluti n are seen in the vicinity of a disintegrating nucleus Several lamellar structures are found inside the nuclear substance Magnification 20000

and even inside the nucleus Some of these mitochondria seem to be ne r complete destruction

In the lumen of the e r of most fibroblasts some dense material has been found The author it not able to give an opinion as to the nature of this material

The intercellular space of the prednisone treated group is greatly reduced so much so that it often appears as narrow slits between inter digitating fibroblasts (Fig 7) In these narrow spaces very few collagen fibrils are seen Such fibrils however, occur in great numbers in lacunae practically devoid of cells In granulation tissues of animals treated with salicylate or phenylbutazone the extracellular space is filled with cellular debris whereas the distance between individual cells is not different from that found in granulation tissue of control animals As in the group treated with prednisone great amounts of collagen fibrils are found in lacunae with rather few cells Thus in all 3 groups collagen formation does not seem to be depressed the fibrils only being arranged in an abnormal way

DISCUSSION

As demonstrated in this paper maturation of the fibroblasts involves formation of an e r composed of parallel membranes studded with RNA

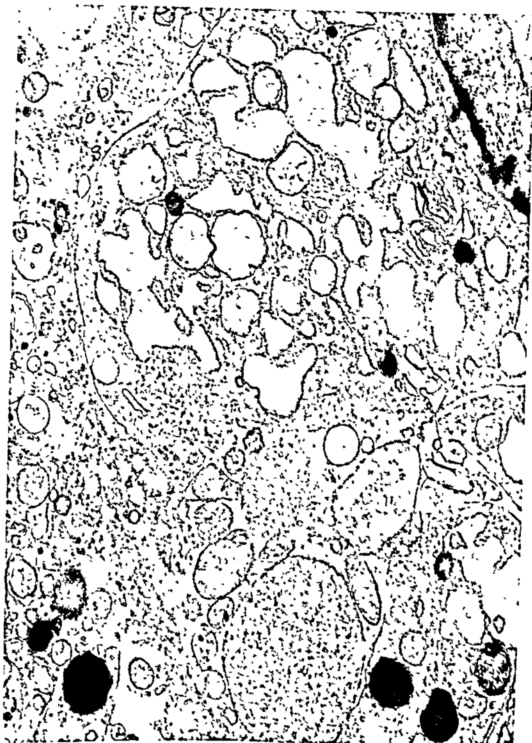


Fig 10

Fibroblasts of salicylate-treated group in partial destruction Dilatation of the e r and of the mitochondria. Several mitochondrial precursors Magnification, 24000



F g 11

Deta l f m f I r I last tr ated th phenylbutazone A gr up of granular I d es an l m to hond a n partial r solut on are seen in the vicin ty of a d s ntegrat ng nucleus Se eral lamellar structures are found ins de the nuclear substance Magnif cation 20000

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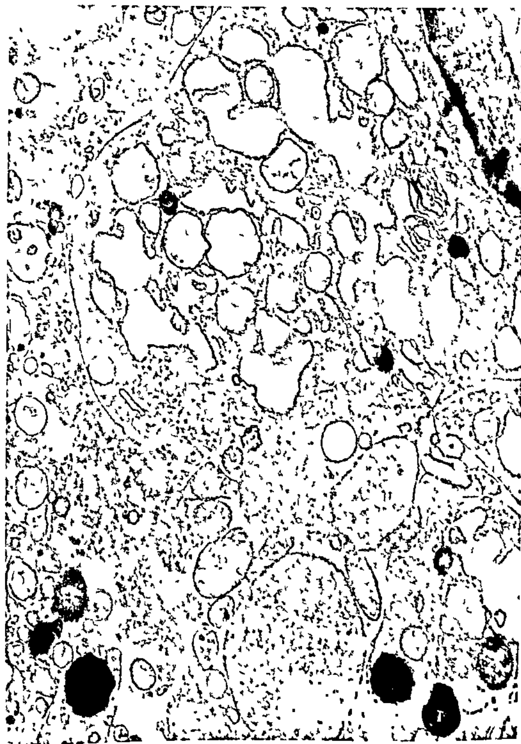


Fig 10

Fibroblasts of salicylate treated group in partial destruction Dilatation of the e r and of the mitochondria Several mitochondrial precursors Magnification 24000

tions of collagen fibrils are found in lacunae without apparent contact with cells rather supports the theory that collagen is secreted from the cell as molecular units with the subsequent aggregation of such units in the extracellular space It also seems of significance that no destruction of cell membranes has been noticed in fibroblasts of control animals The presence of fine fibrillar structures within cells is no proof that such structures take part in normal collagen formation as they may be due to an artifact during preparation The fact that the unbanded fibrils found extracellularly in very young granulation tissue are encountered at some distance from the fibroblasts also is in opposition to the theory that fibrils form in close connection with the cell membrane The unbanded fibrils with a reasonable degree of certainty, are precollagenous as they have been shown to incorporate radioactive proline (*Ross & Benditt* (15)) They are further found in great amounts in granulation tissue of scorbutic guinea pigs, disappearing after administration of ascorbic acid (*Jorgensen* (8), *Ross & Benditt* (14), *Ross & Benditt* (16))

It is remarkable that collagen formation as revealed in the present study as well as in biochemical and light microscopical studies (*Jorgensen* (6, 7)) proceeds normally in spite of the considerable changes of cellular morphology demonstrated in this paper This may indicate that collagen formation—at any rate when ascorbic acid is not lacking—is a very basal function of the fibroblast which is not easily disturbed

In light microscopical investigations (*Jorgensen* (7)) it was found that treatment with both prednisone, phenylbutazone, and sodium salicylate resulted in a reduction of the amount of intercellular substance of granulation tissue This finding has been confirmed in the present investigation as far as treatment with prednisone is concerned As demonstrated here the reduction of intercellular substance due to treatment with phenylbutazone and salicylate is not real, however, the previous findings being due to the accumulations of cell [illegible]

[illegible] *Jorgensen*'s (7) finding that the intensities of the Hale and alcian blue reactions were decreased significantly in granulation tissues of animals treated with prednisone whereas treatment with salicylate and phenylbutazone did not result in this effect

SUMMARY

Complex changes have been found in fibroblasts of granulation tissue of animals treated with prednisone, salicylate, and phenylbutazone It is remarkable that in spite of these morphological alterations collagen formations takes place at a normal rate

granules In later stages dilatation of the e r takes place accompanied by disappearance of the granules characteristic of the active reticulum According to *Peach et al* (11) fibroblasts with an e r with elongated profiles and distinct cell borders are mainly migratory of character, secretory fibroblasts being characterized by the presence of indistinct cell borders and a dilated e r As, however, collagen formation is taking place rapidly in the tissue investigated here without the presence of fibroblasts of the secretory type described by *Peach et al*, no strict distinction between migratory and secretory fibroblasts based on the morphological criteria of these authors seems warranted

The ultrastructure of the cytoplasm of the fibroblasts treated with prednisone in many ways resembles that of younger cells of the control group This may be taken as an indication of an arrested maturation The general architecture of the tissue of prednisone-treated animals does not, however, resemble that found in very young tissues of normal animals, and there is little doubt that the explanation of the effect of prednisone on granulation tissue formation demands further study

The great morphological changes of the ultrastructure of the mitochondria due to treatment with salicylate or phenylbutazone may be part of the general cellular destruction The altered morphology and the accumulation of the mitochondrial precursors may, however, be explained by a more specific mechanism as both salicylate and phenylbutazone are able to uncouple oxidative phosphorylation (*Adams & Cobb* (1), *Jeffrey & Smith* (5)) At the present time nothing more precise can be said of the possible connection between mitochondrial destruction and cellular disintegration A clue to the question may be found in Fig 11 The nuclear destruction seen in this figure is accompanied by an accumulation of partly destructed mitochondria and mitochondrial precursors In the interior of the nuclear substance several lamellar structures are found—possibly mitochondria near complete destruction The possibility exists that during disintegration of these organelles enzymes are released that brings about nuclear and perhaps also cellular destruction In other experimental conditions changes of mitochondrial morphology of a certain resemblance to the lamellar structures found in this study have been described Thus both in the kidney of starved summer frogs and in liver cells of thyrotoxic rats mitochondria with concentric or longitudinal cristae have been found (*Grenawalt et al* (4), *Karnovsky* (9)) The possibility exists that the lamellar structures found in the present study only represent a further development of identical character

In the present investigation no evidence has been found that shedding of fibrillar material is an important step in collagen formation which has been suggested by several investigators, nor has the presence of fibrillar condensations adjacent to the cell membranes been demonstrated (conf *Chapman* (2), *Gieseking* (3), *Karrer* (10), *Porter & Pappas* (12)) The fact that in the treated groups considerable accumula-

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In light microscopical investigations (*Jørgensen* (7)) it was found that treatment with both prednisone phenylbutazone and sodium salicylate resulted in a reduction of the amount of intercellular substance of granulation tissue This finding has been confirmed in the present investigation as far as treatment with prednisone is concerned As demonstrated here the reduction of intercellular substance due to treatment with phenylbutazone and salicylate is not real however the previous findings being due to the accumulations of cellular debris on the extracellular space including closely packed cells The fact that only treatment with prednisone results in a reduction of the amount of intercellular substance also explains *Jørgensen* s (7) finding that the intensities of the Hale and alcian blue reactions were decreased significantly in granulation tissues of animals treated with prednisone whereas treatment with salicylate and phenylbutazone did not result in this effect

SUMMARY

Complex changes have been found in fibroblasts of granulation tissue of animals treated with prednisone salicylate and phenylbutazone It is remarkable that in spite of these morphological alterations collagen formations takes place at a normal rate

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FROM THE RHEUMATIC RESEARCH LABORATORY, UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY, COPENHAGEN (PROFESSOR G TEILUM, M D)

ELECTRON-MICROSCOPICAL STUDIES OF GRANULATION TISSUE FORMATION IN OPEN WOUNDS OF ASCORBIC ACID DEFICIENT GUINEA PIGS

By

OLE JØRGENSEN

Received 16 vii 63

The deleterious effect of ascorbic acid deficiency on wound healing is well established but only a few investigations exist dealing with electron microscopical changes of granulation tissue of scorbutic animals The present investigation aims at investigating the effect of ascorbic acid deficiency on the ultrastructure of granulation tissue of open wounds

MATERIAL AND METHODS

Female guinea pigs weighing 300 g were used Ascorbic acid deficiency was produced as previously described (*Jørgensen* (6)) Control animals received 50 mg of ascorbic acid intraperitoneally every other day Part of the animals deprived of ascorbic acid received 50 mg of ascorbic acid on the last two days before being killed Levo thyroxine was injected as previously described [illegible]

RESULTS

In normal granulation tissues the fibroblasts are seen surrounded by many collagen fibrils (Fig 1) The fibroblasts contain a well developed endoplasmic reticulum (e r) with a predominant parallel arrangement Many dilatations of the e r are found, however On the outer side of the e r great amounts of evenly distributed RNA granules are seen Some free granules (ribosomes) are found in the cytoplasm outside the e r In the cytoplasm fibrillar structures may be seen The mitochondria appear moderately dilated (Figs 2–3)

This investigation was aided by a grant from the *Danish National Association against Rheumatic Diseases* Part of the investigation was carried out in the Laboratories for Investigation of the Pharmacology of the Connective Tissue at the Department of Pharmacology

The author is thankful to *F Carlsen M Sc University Institute of Biophysics* for kind permission to use the *electron* microscope of the institute

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12 [illegible] Collagen Formation [illegible] Fibroblasts of the Chick [illegible]

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14 [illegible] Fine Struc [illegible]

15 [illegible] Wound Healing and Collagen Formation 3 A Quanti [illegible] the Utilization of Proline H^3 in Wounds [illegible]

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FROM THE RHEUMATIC RESEARCH LABORATORY, UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY, COPENHAGEN (PROFESSOR G. TEILUM, M.D.)

ELECTRON MICROSCOPICAL STUDIES OF GRANULATION TISSUE FORMATION IN OPEN WOUNDS OF ASCORBIC ACID DEFICIENT GUINEA PIGS

By

OLE JØRGENSEN

Received 16 vii 63

The deleterious effect of ascorbic acid deficiency on wound healing is well established but only a few investigations exist dealing with electron microscopical changes of granulation tissue of scorbutic animals. The present investigation aims at investigating the effect of ascorbic acid deficiency on the ultrastructure of granulation tissue of open wounds.

MATERIAL AND METHODS

Female guinea pigs weighing 300 g were used. Ascorbic acid deficiency was produced as previously described (*Jørgensen* (6)). Control animals received 50 mg of ascorbic acid intraperitoneally every other day. Part of the animals deprived of ascorbic acid received 50 mg of ascorbic acid on the last two days bef[illegible]

RESULTS

In normal granulation tissues the fibroblasts are seen surrounded by many collagen fibrils (Fig. 1). The fibroblasts contain a well developed endoplasmic reticulum (e.r.) with a predominant parallel arrangement. Many dilatations of the e.r. are found, however. On the outer side of th[illegible] e.r. great amounts of evenly distributed RNA granules are seen. Some free granules (ribosomes) are found in the cytoplasm outside th[illegible] e.r. In the cytoplasm fibrillar structures may be seen. The mitochondria appear moderately dilated (Figs. 2, 3).

Th[illegible]

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[illegible] is thankful to *F. Carlsen, M. Sc., University Institute of Biophysics* for kind permission to use the electron microscope of the institute.

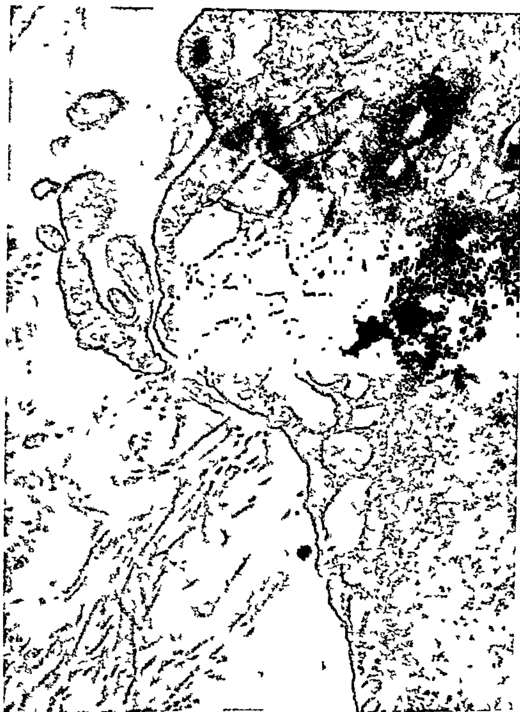


Fig 1

Part of fibroblast from control group with slightly dilated e r with many RNA granules In the extracellular space collagen fibrils are found Magnification 45500



Fig 2

De a l of a f l rol last of the control group with great amounts of sl ghtly d lated granu lar e r A mitochondr on and part of a granular body are s en Magn f cat on 46500

In granulation tissue of ascorb ic acid deficient animals the fibroblasts ar found more scattered than in normal granulation tissue They are surroun l d l y many erythrocytes and dark masses of fine unbanded f l rils Practically no collagen fil rils are found although in some cases such fil rils may l e seen—often intermingled with masses of fine un l anded fil rils (Fig 4)

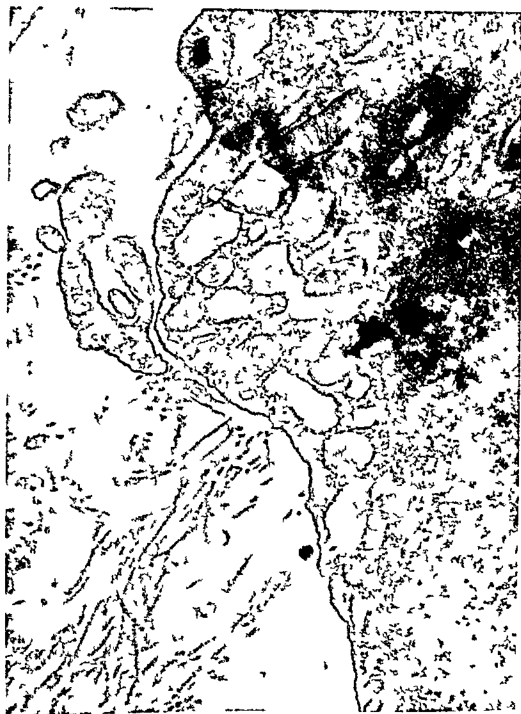


Fig 1

Part of fil rol last from control gro p v ith sl ghtl lilate l e r v th man RNA gra ı ıl s In the extracell ılar space llagen f l ls are f n l Magn f cation 45500



Fig 5

Fibroblasts of scorbutic group with dilated e r, mitochondria of normal appearance and granular bodies In the extracellular space dark masses of unbanded fibrils Among these cross sections of collagen fibrils Magnification 8500

butic fibroblasts Treatment with thyroxine does not result in changes of the ultrastructure of scorbutic granulation tissue (Fig 6)

After treatment for 2 days with ascorbic acid rapid collagen formation takes place in the scorbutic granulation tissue with concomitant disappearance of the unbanded fibrillar material At the same time most of the fibroblasts attain the characteristics of normal fibroblasts (Fig 7)

DISCUSSION

The present investigation confirms the previous findings that the e r of scorbutic fibroblasts is the site of considerable dilatation (*Peach*

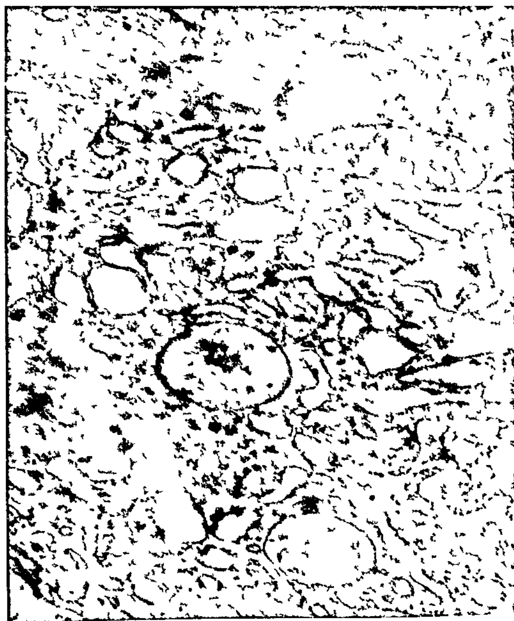


Fig 3
Cytoplasm of a normal fil rol last Cranular e r mitocl on lria part of a Colgi c m plex an l fil rillar stri ctures are seen Magnif cat n 44500

The e r of the scorbutic fibroblasts takes the form of dilated sacs or lacunae on the outer side of which reduced numbers of RNA granules are found In contrast to this great numbers of ribosomes are seen in the cytoplasm outside the e r (Fig 5) The mitochondria of scorbutic fibroblasts appear dilated but not morphologically different from those of normal cells In many cells however increased amounts of granular bodies—believed to be mitochondrial precursors (conf *Jorjensen* (8)) —are found No intracellular fibrillar material has been found in scor



Fig 4

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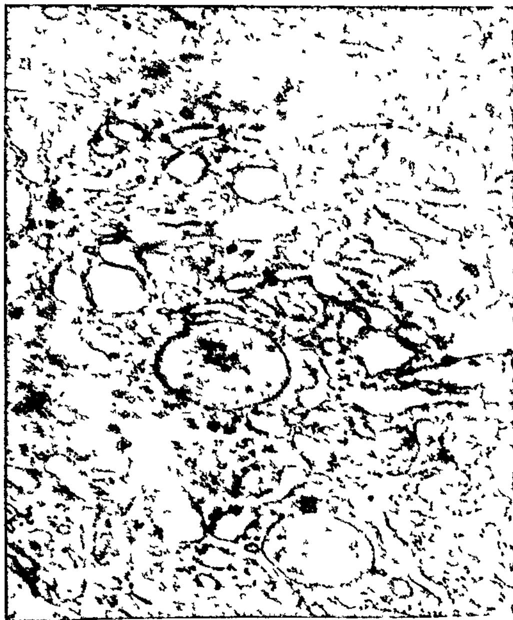


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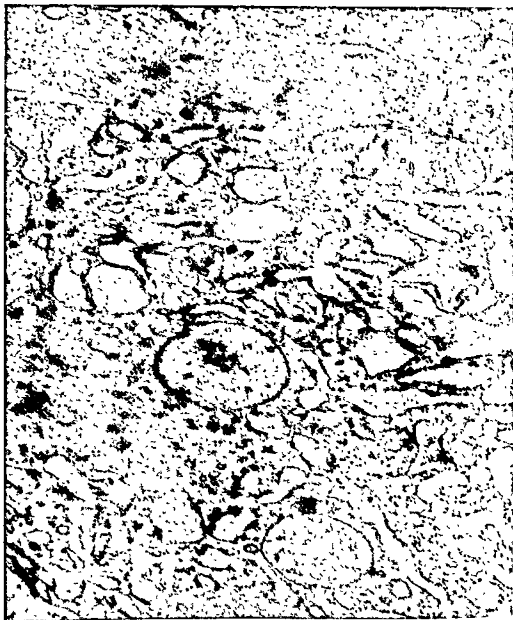


Fig. 3.

Cytoplasm of a normal fibroblast. Granular e r, mitochondria, part of a Golgi complex and fibrillar structures are seen Magnification: 44500.

The e.r. of the scorbutic fibroblasts takes the form of dilated sacs or lacunae on the outer side of which reduced numbers of RNA-granules are found. In contrast to this great numbers of ribosomes are seen in the cytoplasm outside the e r (Fig. 5). The mitochondria of scorbutic fibroblasts appear dilated, but not morphologically different from those of normal cells. In many cells, however, increased amounts of granular bodies—believed to be mitochondrial precursors (conf *Jorgensen* (8)) —are found. No intracellular fibrillar material has been found in scor-



Fig. 6

Fibroblasts of scorbutic group treated with thyroxine. The fibroblasts contain a dilated e.r. and are surrounded by masses of unbanded fibrillar material and erythrocytes. No collagen fibrils are found. Magnification 5000.

found in the cytoplasm of the scorbutic fibroblasts. The significance of these findings is not quite clear. As the e.r. is presumed to be responsible for protein synthesis (*Campbell* (1)) morphological changes of this structure might be the cause of the impaired collagen formation of scorbutic granulation tissue. The same conclusion might be drawn from the finding that normalization of the ultrastructure of scorbutic fibroblasts takes place accompanied by collagen formation when scorbutic animals are treated with ascorbic acid. As in other experimental con-

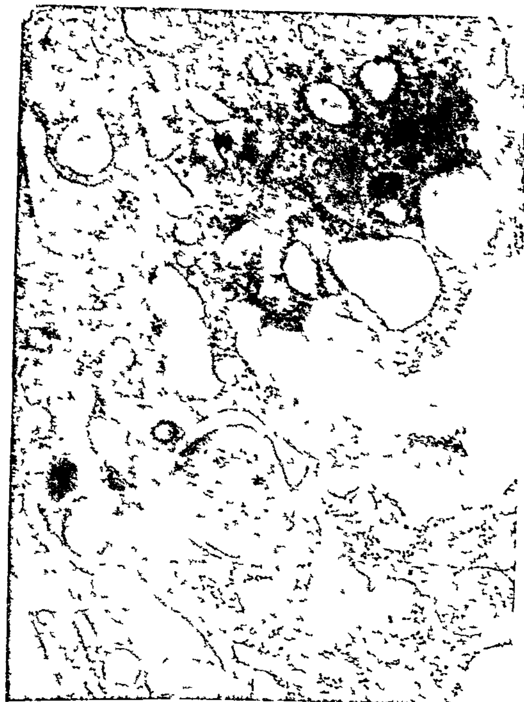


Fig 5

Part of scorl it c f l rol la t (reatl l lated e r tl re l l n ml er f RNA gran les Increa e l am unt f r l me are n n th t p la m t i le the e r Magn f at n 4 0 0

(9) *Ross & Bendall* (11)) In contrast to previous investigators this author finds reduced numbers of RNA granules on the er and increased amounts of ribosomes in the ext plasm outside the er In addition to these changes accumulations of mitochondrial precursors are

normal granulation tissue the label is subsequently found over the collagen fibrils, in the scorbutic granulation tissue over unbanded fibrillar material of the same nature as that found in the present investigation This must imply that fibroblasts of scorbutic granulation tissues are able to utilize proline for the formation of extracellular fibrillar material which must be considered procollagen As unbanded fibrillar material indistinguishable from that of scorbutic granulation tissue is found in very young granulation tissue of normal rats (*Jorgensen* (8)) the procollagen of young normal granulation tissue and that of scorbutic granulation tissue are morphologically identical (A survey of the biochemical investigations concerning the possible existence of collagen precursors in scorbutic granulation tissue has been given by *Gould et al* (5) The evidence here presented seems to be consistent with the view that the cause of the impaired collagen formation is not only to be sought within the fibroblasts

In a previous investigation (*Jorgensen* (7)) it was shown that administration of thyroxine to scorbutic animals bearing open wounds resulted in a reduction of the amount of waterbinding mucopolysaccharide in the granulation tissue without concomitant changes of the concentration of collagen Treatment with ascorbic acid for 2 days resulted in corresponding changes of the mucopolysaccharide concentration accompanied by an increased production of collagen (*Jorgensen* (6, 7)) If the changes of the mucopolysaccharide concentration due to treatment with thyroxine were accompanied by alterations of the ultrastructure of the fibroblasts this finding might have given some clue as to the intracellular site of mucopolysaccharide production As the ultrastructure of the untreated scorbutic fibroblasts does not differ from that of scorbutic fibroblasts treated with thyroxine no such information can be obtained The identical morphology of the fibroblasts of the two scorbutic groups might indicate that the fibroblasts of both groups produce mucopolysaccharide at the same rate (which is not necessarily different from that of normal fibroblasts) and that the accumulation of hyaluronic acid extracellularly in ascorbic acid deficiency might be due to a lack of some unknown factor in the extracellular space which in normal granulation tissue—is responsible for the removal of this mucopolysaccharide As shown by *Daubenmerkl* (3) a mixture of ascorbic acid and an oxidating agent is able to degradate hyaluronic acid Thus the hypothetical agent responsible for the removal of hyaluronic acid may very well be ascorbic acid itself The possibility must be considered that thyroxine may be able to replace ascorbic acid as regards its degradating effect on hyaluronic acid

The fibroblasts of 8-day old granulation tissue of normal [illegible]

[illegible] that of the rats Furthermore, intracellular fibrillar formations are more abundant in the fibroblasts of guinea pigs

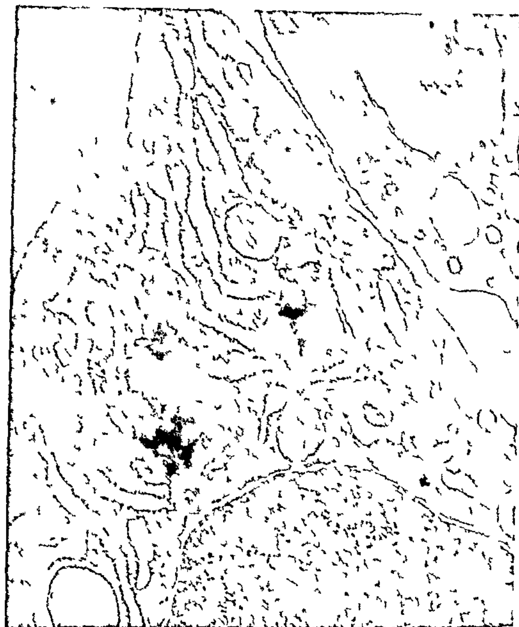


Fig 7

Parts of fibroblasts of ascorbic acid deficient group treated with ascorbic acid The e r f one of the fibroblasts appear quite normal the e r of the other fibroblast still retains its scorbutic appearance Magnification 43500

ditions (*Jorgensen* (8)) collagen formation proceeds *normally in spite* of considerable changes of the e r and even destructions of the fibro blasts it is not safe to conclude however that the changes of the ultra structure of the fibroblasts and the impaired collagen formation re present a cause and effect relationship

As shown by *Ross & Benditt* (12) labelled proline is incorporated into fibroblasts of both normal and scorbutic granulation tissue In

normal granulation tissue the label is subsequently found over the collagen fibrils in the scorbutic granulation tissue over unbanded fibrillar material of the same nature as that found in the present investigation. This must imply that fibroblasts of scorbutic granulation tissues are able to utilize proline for the formation of extracellular fibrillar material which must be considered procollagen. As unbanded fibrillar material indistinguishable from that of scorbutic granulation tissue is found in very young granulation tissue of normal rats (*Jorgensen* (8)) the procollagen of young normal granulation tissue and that of scorbutic granulation tissue are morphologically identical. (A survey of the biochemical investigations concerning the possible existence of collagen precursors in scorbutic granulation tissue has been given by *Gould et al.* (5).) The evidence here presented seems to be consistent with the view that the cause of the impaired collagen formation is not only to be sought within the fibroblasts.

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The fibroblasts of 8 days old granulation tissue of normal guinea pigs exhibit certain characteristics distinguishing them from rat fibroblasts of the same age (conf. *Jorgensen* (8)). Thus the e. r. of the guinea pigs appears more dilated than that of the rats. Furthermore intracellular fibrillar formations are more abundant in the fibroblasts of guinea pigs

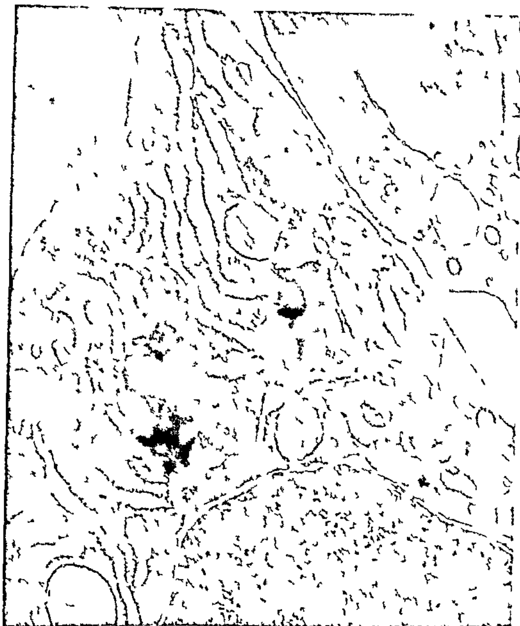


Fig 7

I arts of f I rol la ts of a rl c c l lef c ent gr I treate I th a rl c ac l The e r of ne of tl e f I rol la t apj ears q te n rmal the e r f th ther f I rol la t st ll reta ns its corl ut apj earance Magnif cat on 43 00

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13 *Rudas R* Zur quantitativen Bestimmung von Granulationsgewebe in experimentell erzeugten Wunden Arzneimittelforsch *10* 226–229 1960

than in those of the rats. This does not necessarily mean that the mechanism of collagen formation is different in the two species. Some authors are of the opinion that collagen fibrils may be formed by shedding of intracellular fibrillar material (*Chapman* (2), *Gieseking* (4)) or in close connection with the cell membrane in the extracellular space (*Porter & Pappas* (10)), but no definite proof of such theories exists.

The fact that the procollagenous fibrils both in scorbutic and in very young granulation tissue of normal animals (conf. *Jorgensen* (8)) are found at some distance from fibroblasts supports the theory that fibrillar collagen is formed in the extracellular space after secretion of procollagen units invisible in the electron microscope. The same conclusion may be drawn from the finding that in animals treated with antirheumatic compounds collagenous fibrils are principally found in lacunae free of cells whereas very few fibrils are seen where aggregations of cells occur (*Jorgensen* (8)).

SUMMARY

The ultrastructure of scorbutic granulation tissue is characterized by the presence of erythrocytes and unbanded fibrillar material in the extracellular space. The fibroblasts contain a greatly dilated endoplasmic reticulum with reduced numbers of RNA-granules. In the cytoplasm outside the endoplasmic reticulum increased amounts of ribosomes are seen.

It is proposed that factors in the extracellular space may be of importance to the impaired collagen formation as well as to the accumulation of acid mucopolysaccharides in scorbutic granulation tissue.

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FROM STATENS SERUMINSTITUT, COPENHAGEN (DEPT OF DIAGNOSTIC BACTERIOLOGY CHIEF H LAUTROP, M D)

THE ONPG TEST IN DIAGNOSTIC BACTERIOLOGY

1 *Methodological Investigations*

By

PER BULOW

Received 21 viii 63

Orto-nitrophenyl-β-D-galactoside (ONPG) is a colourless artificial chromogenic enzyme substrate in which one molecule of orto-nitro phenol and one molecule of galactose are united by a β-galactoside bond Lactose is a β-galactoside composed of glucose and galactose

ONPG was prepared in 1950 by *Seidman* and *Link* and was used by *Lederberg* to study β-D-galactosidase in *Escherichia coli* (1950) This enzyme is able to split the β-galactoside bond in both ONPG and lactose (*Cohn & Monod* 1951) The advantage of ONPG is that the yellow compound orto-nitrophenol is liberated by the action of β-galactosidase on the β-galactoside bond Using ONPG to demonstrate the presence of β-galactosidase, one can see and measure directly the activity of the enzyme

ONPG has been used to study β galactosidases in various bacteria, and new information has been obtained concerning these enzymes, the phenomenon of enzyme induction and specific permease systems

It has thus been established that most of the β-galactosidases prepared from various bacteria belonging to the family *Enterobacteriaceae* have identical kinetic properties (*Monod & Cohn* 1952), though their serological properties may vary, as well as their ability to withstand a variety of agents such as heat, urea trypsin, toluene etc (*Anderson & Rickenberg* 1960) *Cohn & Monod* (1951) found that the enzymatic hydrolysis of lactose and ONPG is influenced by monovalent cations and that sodium is more active than potassium in the hydrolysis of ONPG, whereas potassium is more active when lactose is the substrate It is important to keep these observations in mind when trying to use ONPG under the standardized conditions of a practical routine test

Since *Karstrom* (1938) drew attention to the difference between what he called "constitutive and adaptive or inducible enzymes", it has become more and more evident that the substrate induction of enzymes plays a very prominent role in bacteria (*Deere et al* 1939 *Monod* 1947, *Monod et al* 1951, *Rickenberg* 1960 (1)) It has been shown (*Deere et al* 1939, *Lowe* 1960, *Rickenberg* 1960 (2)) that β galactosidase could be

present in bacteria even if they were unable to ferment lactose or were late-fermenters and that the non lactose fermenting organisms were often more or less impermeable for lactose. Induction of the enzyme does not of course occur unless the substrate is transported to the interior of the cell. When the inducer has penetrated the cell membrane the induced enzyme appears after a lag of about 3 minutes (*Pardee & Prestidge* 1960).

Studies by *Rickenberg et al.* (1956) and *Rotman* (1958) have revealed the presence of a galactoside permease system in *E. coli*. This enzyme system is responsible for the transportation of β galactosides through the cell membrane and is not identical with the β galactosidase but is specifically induced—usually together with the β galactosidase by certain galactosides.

Instead of establishing direct contact between the β galactosidase and the substrate inside the cell one can liberate the enzyme from the cell by disintegration. Thus it was found by *Lederberg* (1950) that the β galactosidase activity of cells of *E. coli* was greatly increased by toluene treatment of the cells. Similarly *Rickenberg* (1960 (2)) was able to demonstrate in bacteria belonging to the genus *Shigella* a higher enzymatic activity after treatment with toluene than before. On the other hand it is known that toluene labile β galactosidases exist (*Anderson & Rickenberg* 1960).

These different facts explain why it is necessary when performing the ONPG test as a routine bacteriological examination to include an enzyme inducing substrate in the culture medium and why attempts have been made to enhance the autolysis by the addition of toluene to the cell suspensions.

The present investigation was prompted by a study by *Le Minor & Ben Hamida* (1962) in which they describe their technique of using ONPG in a routine test for demonstrating the presence of β galactosidase in bacteria. Before adopting their technique it was found necessary to study some aspects of it in greater detail.

Le Minor & Ben Hamida's technique is as follows: suspend in 0.25 ml of saline a loopful of a bacterial culture from a lactose glucose SH-medium that has been incubated for 18 hours at 37° C, add one drop of toluene and agitate the suspension at 37° C for some minutes, then add 0.25 ml ONPG buffer solution and place the test tube in a waterbath at 37° C, take readings after 20 minutes, 1, 2, 3 and 20 hours of incubation.

INVESTIGATIONS

1. *Material*

The strains used in the present investigation include a minor part of a material [illegible] of 1012 strains of Gram negative rods, 579 of them belonging to the family *Enterobacteriaceae*, 343 to the family *Pseudomonadaceae* and the remainder to other families.

Further information about this material will appear in a subsequent paper.

2 Reagents

For the *buffer solution* the following formula was used

$NaH_2PO_4 \cdot H_2O$	6 9 gram
Dissolve in distilled water	40 ml
Adjust to pH 7 0 with NaOH 5N about	7 5 ml
Add distilled water to a final volume of	50 ml

The solution is stored at 4° C Under this condition crystallization may take place so the solution should be heated for a few minutes at 37° C before use to redissolve any crystals

The pH of the buffer was chosen in accordance with the experiments by *Lederberg* (1950), who found that the pH optimum for β galactosidase in cellular extracts of *E coli* was 7 3 whereas the optimum was about 7 0 and the activity curve more flat when intact cells were used Similar results were found with *Aerobacter aerogenes Shigella sonnei* and *Paracolobactrum aerogenoides* by *Monod & Cohn* (1952) and *Anderson & Rickenberg* (1960)

The formula for the *ONPG solution* was

Crystalline ONPG (L Light & Co Ltd Colnbrook England	80 mg
Dissolve in distilled water	15 ml
Add buffer solution	5 ml

The final reagent has a molarity of 0 25 with respect to the phosphate buffer As o nitrophenol is weakly acid and an accumulation of acid or alkaline products may take place in the bacterial culture, the ONPG solution has to be highly buffered

The ONPG solution is not quite stable as evidenced by a weak yellow colour developing after storage for some time at 4° C It has been observed that various agents—for instance traces of zinc or of course contamination with bacteria containing β galactosidase—promote the hydrolysis considerably A yellow reagent should not be used

3 Density of the Bacterial Suspension

Le Minor & Ben Hamida recommend the use of very dense bacterial suspensions obtained by taking a standard platinum loop filled with plenty of culture from the surface of a solid medium

Colony-counting showed that suspensions prepared in this way ("routine-suspensions') have a density of about 10^{11} cells per ml (three strains of *E coli*, one of *Proteus morgani* and one of *P aeruginosa* were counted), comparison of the optical density confirmed that these suspensions did not show much variation

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Dilution of the suspension	1 1	1 2	1 4	1 8	1 16	1 32	
Fast reacting ONPG positive coli strain	5 10	5 10	5 10	20	20	60	minutes
Slow reacting ONPG positive coli strain	120	120	180	1200	>1200	>1200	minutes

The relation between the density of the suspension and the time lapse before the reaction became positive was determined with two strains of *E coli* which differed considerably with respect to this time lapse The results (see Table 1) demonstrate that in both strains there is a definite correlation between density and time lapse

At the same time 25 different routine suspensions were prepared from each of the two cultures and the time lapse before positive reaction was determined It was found that all suspensions became positive within a period corresponding to that required by the three first density steps in the dilution series (Table 1)

These observations confirm that very dense suspensions as used by *le Minor & Ben Hamida*, can be reproduced with sufficient accuracy and show the advantage of a dense suspension over a more dilute one, for the purpose of a routine test

4 *The Suspension Medium*

Ten fast reacting and two slow reacting ONPG positive strains of *F coli* were used to examine the influence of the suspension medium on the course of the ONPG reaction The strains were suspended in distilled water in 0 9 or 2 0 per cent solutions of sodium chloride

It was impossible to demonstrate any differences in the time or the intensity of the ONPG reaction in the different media

5 *Addition of Toluene to the Suspension*

One feature of the technique described by *le Minor & Ben Hamida* was the use of toluene pour favoriser la liberation de l enzyme , but this function does not seem to be quite clear

It has already been mentioned that the addition of toluene to a suspension of intact cells sometimes promoted the hydrolysis of ONPG (*Lederberg* 1950 *Rickenberg* 1960) sometimes an inactivation of the β galactosidase (*Anderson & Rickenberg* 1960) The literature deals with bacterial cells of *F coli Shigella* and *Paracolobactrum aerogenoides* (in this paper the latter is called *Serratia alba*)

Under carefully standardized conditions of the ONPG test a comparison was made between toluene treated and non treated cells from 15 strains representing the three above mentioned kinds of bacteria This investigation did not reveal any significant difference in the time for the appearance of the yellow colour

The ONPG test was also performed both with and without the addition of toluene using 700 strains of Gram negative rods out of the total material of 1012 strains Neither did this comparison reveal any significant promotion of the reaction by the addition of toluene

If a reaction became positive within 3 hours it was neither promoted nor delayed by the addition of toluene

A weak colouring will now and then appear in the toluene free tubes

2 *Reagents*

For the *buffer solution* the following formula was used

$NaH_2PO_4 \cdot H_2O$	6 9 gram
Dissolve in distilled water	40 ml
Adjust to pH 7 0 with NaOH 5N about	7 5 ml
Add distilled water to a final volume of	50 ml

The solution is stored at 4° C Under this condition crystallization may take place so the solution should be heated for a few minutes at 37° C before use to redissolve any crystals

The pH of the buffer was chosen in accordance with the experiments by *Lederberg* (1950) who found that the pH optimum for β galactosidase in cellular extracts of *E coli* was 7 3 whereas the optimum was about 7 0 and the activity curve more flat when intact cells were used Similar results were found with *Aerobacter aerogenes Shigella sonnei* and *Paracolobactrum aerogenoides* by *Monod & Cohn* (1952) and *Anderson & Rickenberg* (1960)

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after about 20 hours, whereas the tubes containing toluene remain uncoloured. This late type of reaction seems to be quite non-specific and is apparently inhibited by toluene.

When the test tubes are incubated for more than 3 hours and especially at the reading after 20 hours, the yellow colour (representing a positive ONPG reaction) is often more intense if toluene has been added. This observation probably means that the cell membranes of the bacteria concerned not always permit unrestricted passage of ONPG from the environment to the interior of the cell. The liberation of enzyme caused by cell-disintegration due to toluene is probably a process requiring some time (i. e. more than 3 hours).

Ten fast-reacting and two slow-reacting ONPG-positive strains of *E. coli* (adapted to lactose) were used to compare the influence of various other lipid solvents: benzene, xylene, ether and chloroform.

All five substances appeared to delay the development of the yellow colour. The delay caused by benzene, toluene and xylene, however, was negligible; ether and especially chloroform caused an appreciable delay of the reaction, most strikingly demonstrated in the two slow-reacting coli strains.

Accordingly, it was decided not to use toluene in the routine test.

6. *Conditions of Incubation*

In all cases the ONPG hydrolysis was carried out at 37° C. A comparison was made between incubation in an air-incubator and in a waterbath, using five slow-reacting strains (one strain each of *E. coli*, *Serratia marcescens*, *Citrobacter freundii*, *Salmonella arizonae* and *Pseudomonas aeruginosa*) and five fast reacting *E. coli* strains.

In the course of the ONPG reaction no differences were observed which might be ascribed to the incubation conditions.

7. *Reading of the Reaction*

In diagnostic bacteriology the ONPG test should be used as a qualitative test. Therefore colorimetric estimation of colour-intensity was not used, and a visual distinction between weak, moderate and strong reactions was made throughout.

Moderate and strong reactions were recorded as positive; the weak and late reactions were regarded as non specific, due to enhanced spontaneous splitting of the substrate, and were therefore recorded as negative.

During the preliminary experiments, readings were made after 20 minutes, 1, 2, 3 and 20 hours, unless otherwise stated.

In routine bacteriology it seems convenient to take a first reading after 20 minutes. If a yellow colour has developed at this time, the reaction is recorded as positive. If no colour is visible after 20 minutes the test tubes should be reinspected after 3 hours of incubation. If the

tube is then still uncoloured, it means there is little likelihood that a significant yellow colour will develop later on, so for practical purposes the reaction may be recorded as negative

This statement is based on an investigation of about 1000 strains of Gram negative rods, to be described in a subsequent paper

8 *Composition of the Growth Medium*

During this investigation the following 1 8 per cent agar media were used

(a) A minimal substrate containing one per cent of $NH_4H_2PO_4$ and 0 5 per cent of glucose
(b) A broth base composed of oxheart infusion broth with one per cent of peptone
(c) The broth base with one per cent of lactose
(d) The broth base with ten per cent of lactose

The two lactose containing media used to demonstrate acid production are (1) an aqueous solution containing one per cent of peptone, 0 3 per cent of Liebig s meat extract, 0 5 per cent of NaCl, and bromthymolblue added as an indicator and (2) Hugh & Leifson s (1953) "oxidation fermentation medium containing one per cent of lactose

To examine the influence of the lactose concentration in the growth medium for the demonstration of β galactosidase, a few induction experiments were carried out

Twenty eight strains of *E coli* which all formed acid from lactose within two days were tested for their ability to split ONPG after cultivation on the minimal substrate, on the broth base and on the broth base with one per cent of lactose

The results are given in Table 2

TABLE 2
The Influence of Substrate Composition on the ONPG Reaction Using 28 Lactose Positive Strains of E coli

Time in minutes before a positive ONPG reaction appears	5	10	20	60	120	180
Minimal substrate	0	0	2	26	26	27
Broth base	1	1	27	27	27	28
Broth base + 1 per cent of lactose	27	27	28	28	28	28

The figures indicate the number of strains that were recorded as ONPG positive at the indicated times

It [illegible]
broth [illegible]
sidas[illegible] [illegible]ated in 27 out of 28 coli strains within 20 minutes by the ONPG reaction In one strain the enzyme was not

revealed until after 3 hours. After cultivation on the broth base with one per cent of lactose, all strains became ONPG-positive within an incubation period of 20 minutes.

In view of *Monod's* original observation (1941)—later confirmed by *Cohn & Horibata* (1959)—showing that glucose inhibits the induced formation of β-galactosidase, it might be objected that glucose ought not to be included in the minimal substrate; however, it was found that on the glucose-containing medium 27 out of 28 strains of *E. coli* became ONPG-positive within 3 hours. Even if the cells are adapted to lactose before inoculation on the minimal substrate, the acquired enzyme system is diluted out after 4–6 generations or inactivated in some way in the absence of inducer (*Cohn* 1957). The minimal substrate was included in the experiment so as to obtain non-induced cells as a basis for comparison.

Two late lactose-positive coli strains (forming acid after 5 and 23 days respectively) and two lactose-negative coli strains (observed for 40 days) were tested for their β-galactosidase content after they had been grown for 22 hours on the minimal substrate, on the broth base, and on the broth base with one or with ten per cent of lactose.

The findings are indicated in Table 3.

TABLE 3

The Influence of the Substrate Composition on the ONPG Reaction Using 2 Late-Lactose Positive and 2 Lactose Negative Strains of E. coli

Time in minutes before a positive ONPG reaction appears	5	10	20	60	180
Minimal substrate					○●
Broth base				○●	
Broth base + 1 per cent of lactose		○●		△	▲
Broth base + 10 per cent of lactose	○●		△	▲	

○● are the signatures for 2 individual late lactose positive strains of E. Coli.
△▲ are the signatures for 2 lactose negative coli strains.

Table 3 shows that in the two late lactose positive coli strains β-galactosidase was revealed within 3 hours, even when they had been grown on a lactose free medium, and that the appearance of a positive ONPG reaction can be promoted considerably by the addition of lactose to the medium. The difference between the two strains as seen in the "fermentation tube" was not revealed by the ONPG reaction under these experimental conditions.

The two lactose-negative coli strains were ONPG positive after 3 hours when using the broth base with one per cent of lactose. The addition of ten per cent of lactose promoted the reaction, and the test was read as positive after 20 and after 60 minutes. Cultivated on the minimal substrate, they remained ONPG-negative for an observation period of 20 hours.

According to Table 3 it would seem reasonable to recommend for routine purposes a lactose concentration of 10 per cent in the growth medium This high concentration, however, is inconvenient for several reasons e g growth is not infrequently inhibited to a considerable extent it is also difficult to dissolve the great amount of sugar, which makes the preparation of the agar medium difficult

A high lactose-content in the growth medium was only found of significant importance in 7 out of 30 strains of *Citrobacter freundii*, taken from a total of about 1000 strains of Gram negative rods dealt with in a subsequent paper

It is not very probable that a lactose concentration between one and ten per cent would be of any value in the process of induction, for the following reasons (1) the permease-positive organisms can accumulate β galactosides intracellularly even if the concentration in the environment is very low, and in this way the enzyme synthesis is stimulated to the maximum by the one per cent of lactose, (2) the enzyme-forming system for β galactosidase in the permease-negative cells does not increase in amount in response to inducer (*Herzenberg* 1959) The only way lactose can be transported from the environment to the interior of a permease-negative cell is probably by passive diffusion, which means that the concentration in the media has to be very high and non physiological

9 *Growth Temperature*

The majority of the strains were cultivated at 35° C Strains belonging to the genus *Pseudomonas*, however, were grown at 22° C

Out of a material of 30 strains of *Hafnia*, 5 strains were found ONPG-negative, provided they were grown at 35° C, grown at 22° C (for 22 hours), 3 out of 5 strains became ONPG-positive within 20 minutes At the low growth temperature the 3 strains were persistently positive even if they had been grown on the broth base without lactose

It may be added that in the conventional lactose fermentation test the 3 strains were not influenced by the low temperature either, but remained negative throughout an observation period of 30 days

At a growth temperature of 35° C or 37° C, all the 5 *Hafnia* strains (i e those that were found ONPG negative in the initial experiment) remained negative, even if the lactose concentration in the growth media was increased

This observation proves that a low growth temperature may be of decisive importance for demonstrating the presence of β-galactosidase by the ONPG test However, this situation is probably rather an uncommon one so in routine bacteriology it is hardly necessary to pay attention to it except for some special reason e g a suspicion that the strain belongs to the species *Hafnia alvei*

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10 *Duration of Growth Period*

The effect of varying the growth period on the course of the development of a positive ONPG reaction was examined, using a fast-reacting ONPG-positive coli strain and a slow-reacting one; the latter was lactose-negative, which in this case probably means "permease-negative" The broth base with one per cent of lactose was used as a growth medium

The findings appear in Table 4

TABLE 4
Effect of Duration of Growth Period on the Course of the ONPG Reaction

Duration of growth period in hours	12	18	24	36	48	60	
Fast-reacting coli strain	10	5–10	5–10	5–10	5–10	5–10	minutes
Slow-reacting coli strain	240	180	120	120	120	120	minutes

The figures indicate the time taken for a positive ONPG reaction to develop

The duration of the growth period seems to be of some importance for the development of a positive ONPG reaction, this is probably linked up with the fact that the process of induction may require some time under the present conditions

An ONPG-positive strain of *Pseudomonas aeruginosa* was tested in the same manner, but in this case the growth period had no demonstrable influence on the development of the positive ONPG reaction This observation agrees with the assumption (discussed in a subsequent paper) that the β-galactosidase active principle present in the ONPG-positive strains of *P aeruginosa* is not an inducible one

11 *Repeated Subcultures*

If induction of demonstrable β-galactosidase was not possible with growth media containing 10 per cent of lactose, repeated subcultivations were performed on media containing the same high lactose concentration, to find out whether that would affect the course of the *ONPG* reaction

The results of these experiments (further dealt with in a subsequent paper) showed that repeated subcultivation on lactose media did not promote the ONPG reaction

12 *Description of the Standard Technique, and a Proposal for a Minor Modification*

So far the individual steps in the ONPG test have been examined and certain information which may be useful in the practical performance

of the test has been collected As a result of these experiments a suitable method for the performance of the ONPG test can now be described

A platinum loop is filled with plenty of bacterial culture from the surface of a broth agar plate containing one per cent of lactose incubated for 18–22 hours at 35–37° C A heavy suspension is made in 0 25 ml sterile distilled water (or saline) and 0 25 ml ONPG reagent is added The tubes are incubated at 37° C in an incubator or a waterbath, and the first reading is taken after 20 minutes If the reaction is negative, the reading should be repeated after an incubation period of three hours If the suspension is still uncoloured, the strain should be designated as ONPG negative A yellow colour after 20 minutes or three hours means that the strain is ONPG positive or—if it is preferred—β galactosidase positive

It might however, be expedient to introduce a minor modification of the test for routine identification purposes Instead of mixing equal amounts of bacterial suspension and ONPG reagent it would facilitate the procedure to suspend the bacteria directly in the ONPG reagent diluted twofold

This proposed routine method was compared with the above mentioned standard technique The five fast reacting and the five slow reacting strains which were used in section 6, were employed in the experiment The ONPG reagent dilution (1 1) was tried immediately after preparing the reagent after one and after two weeks' storage at 4° C

There were no variations in the time of the appearance of a positive ONPG reaction or its strength that could have been accounted for by the proposed modification

SUMMARY

Some details concerning the standardization of the ONPG reaction as a practical routine test for the demonstration of β galactosidase in Gram negative rods have been studied

On the basis of these studies a description of the standard technique is given together with proposal for a minor modification for the routine use of the test

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Effect of Duration of Growth Period on the Course of the ONPG Reaction

Duration of growth period in hours	12	18	24	36	48	60	
Fast reacting coli strain	10	5–10	5–10	5–10	5 10	5–10	minutes
Slow reacting coli strain	240	180	120	120	120	120	minutes

The figures indicate the time taken for a positive ONPG reaction to develop

The duration of the growth period seems to be of some importance for the development of a positive ONPG reaction, this is probably linked up with the fact that the process of induction may require some time under the present conditions

An ONPG positive strain of *Pseudomonas aeruginosa* was tested in the same manner, but in this case the growth period had no demonstrable influence on the development of the positive ONPG reaction This observation agrees with the assumption (discussed in a subsequent paper) that the β galactosidase active principle present in the ONPG positive strains of *P aeruginosa* is not an inducible one

11 *Repeated Subcultures*

If induction of demonstrable β-galactosidase was not possible with growth media containing 10 per cent of lactose, repeated subcultivations were performed on media containing the same high lactose concentration, to find out whether that would affect the course of the ONPG reaction

The results of these experiments (further dealt with in a subsequent paper) showed that repeated subcultivation on lactose media did not promote the ONPG reaction

12 *Description of the Standard Technique, and a Proposal for a Minor Modification*

So far the individual steps in the ONPG test have been examined and certain information which may be useful in the practical performance

FROM STATENS SERUMINSTITUT COPENHAGEN (DEPT OF DIAGNOSTIC BACTERIOLOGY CHIEF H LAUTROP M D)

THE ONPG TEST IN DIAGNOSTIC BACTERIOLOGY

2 *Comparison of the ONPG Test and the Conventional Lactose-Fermentation Test*

By

PER BÜLOW

Received 21 viii 63

The purpose of the present paper is to examine the value of the ONPG test in diagnostic bacteriology, and for this purpose the ONPG test has been compared with the conventional lactose fermentation test, using a material of about 1000 strains of Gram negative rods

ONPG was used by *le Minor & Ben Hamida* (1962) in a similar way to test 586 strains of Gram negative rods for the presence of β galactosidase They found the ONPG reaction convenient in diagnostic bacteriology because it directly reveals the presence of an enzyme which acts on the first link of a chain of enzymatic reactions, they also found that it was possible to evade partly the permease system by the use of toluene treatment of the cells The presence of β galactosidase in late-lactose fermenters was easily demonstrated, and the authors propose that the ONPG test should be used as a supplementary test if the bacterial culture is lactose negative after an incubation period of 18 hours

Mollaret & le Minor (1962) used the ONPG test in differentiation within the genus *Pasteurella*, Leclerc (1962) used it in an investigation of pigmented *Enterobacteriaceae*

The same test was used by *Szturm Rubinsten* (1962) in an investigation of the *Alcalescens Dispar* group because of the var[illegible]

[illegible] strains were ONPG negative, too *Szturm Rubinsten & Piechaud* (1963) examined 419 *Shigella* strains and found indisputable positive ONPG reactions within *Sh dysenteriae* serotype 1, *Sh sonnei* biotype a and *Sh boydii* serotype 9 biotype [illegible] Positive reactions were not found in the *Sh flexneri* subgroup or in other serotypes of *Sh dysenteriae* and *Sh boydii*

MATERIAL

The investigation comprises 1012 strains of Gram negative rods Of this total 579 strains belong to the family *Enterobacteriaceae* 343 to the family *Pseudomonadaceae* and the remainder to other families

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Escherichia coli

Thirty two strains were examined After cultivation on the broth agar containing one per cent of lactose 30 strains were ONPG positive within 20 minutes Twenty eight strains of these formed acid from lactose within one or two days two strains did not become lactose positive until after 5 and 23 days respectively and two strains remained lactose negative throughout an observation period of 30 days The two last mentioned strains were ONPG positive within one and three hours respectively

Escherichia dispar and alcalescens

Nine strains of *E dispar* were all ONPG positive within 20 minutes Eight strains of these formed acid from lactose within 1 3 days One strain became lactose positive within 8 days of incubation

Six strains of *E alcalescens* were both ONPG negative and unable to form acid from lactose throughout an observation period of 28 days An addition of 10 per cent of lactose to the growth media a cultivation of the strains at 30° C and 22 C and an increase of the number of subcultivations on lactose containing media did not result in a positive ONPG reaction

Shigella dysenteriae serotype 1

Nine strains were all ONPG positive within three hours and 6 of them were positive within 20 minutes Only 3 strains formed acid from lactose within 10 12 days whereas 6 strains were lactose negative for 24 days

Shigella sonnei

Fifteen strains were examined Twelve were promptly ONPG positive and lactose positive within 5 11 days

The remaining strains were both lactose negative and ONPG negative even after an attempt to induce β galactosidase by increasing the concentration of lactose in the media to ten per cent Neither did a change in the growth temperature (30 C and 22° C) have any effect on the induction process

Other Shigella

Included in this series are 13 strains of *Sh dysenteriae* (except typ 1 [illegible]

Th [illegible] for 4 days

An attempt was made to reveal latent β galactosidase by the procedures mentioned in the paragraph on *E alcalescens* but the result was negative

The majority came from the strain collection of the department, 13 type strains of *Shigella dysenteriae* were received from Dr Patricia Carpenter Colindale London, 10 strains of *Salmonella subgenus II* from Dr F Kauffmann Statens Serum institut, Copenhagen[1], and the remainder were fresh isolates supplied from the laboratory for bacteriological routine identification In most cases the identities of the strains were assumed to agree with their labels with no further examination since the majority of them have recently been subjected to a thorough investigation In some groups, however, especially if the results of a minority of strains within a group were at variance with the majority within the same group the diagnosis was confirmed by repeated examinations

The present material is not supposed to represent the true proportions found in nature between ONPG positive and ONPG negative strains within the individual groups examined For that purpose a non selected material would have been required and it is known that some strains from the collection are in fact selected for instance, the 24 strains of *Citrobacter freundii* were originally kept because they did not ferment lactose Other groups may also be selected although it is not stated in the records

METHODS

A previous paper (*Bülow* 1964) deals with a methodological investigation of the ONPG reaction on the basis of which a standard method for the performance of the test has been developed

ONPG test A platinum loop is filled with plenty of bacterial culture which has been grown for 20 22 hours at 35° C on a broth agar medium containing one per cent or ten per cent of lactose The bacteria are suspended in 0 25 ml of sterile distilled water (or saline), 0 25 ml of the ONPG reagent is added The tubes are incubated at 37° C in a waterbath or an air incubator The readings for moderate or strong yellow coloration are made after 20 minutes one and three hours

Certain deviations from the described standard method will be mentioned along with the individual groups of bacteria

Lactose fermentation tests Test tubes containing an aqueous solution of one per cent peptone 0 5 per cent meat extract (Liebig) 0 5 per cent lactose 0 5 per cent NaCl and bromthymolblue as an indicator are inoculated with the bacteria in question and placed in an air incubator at 35° C The tubes are read for acid formation (*i e* colour change of the indicator from blue to yellow) and observed for at least three weeks

This test was used in examining the facultatively anaerobic bacteria while the strict aerobes *i e Pseudomonas* and *Cytophaga* were tested for acid formation in *Hugh & Leifson s* oxidation fermentation medium (1953) containing one per cent of lactose and observed for at least three weeks

RESULTS

The results obtained in the comparison of each group of organisms will now be commented on the findings are summarized in Tables 2 and 3

Special conditions necessary for the demonstration of β-galactosidase are discussed in connection with the relevant groups of bacteria, taxonomic references are given when necessary for an unequivocal designation of the strains

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1 The author wishes to express his gratitude to *Dr Patricia Carpenter* Colindale London for forwarding 13 type strains of *Shigella dysenteriae* and for serotyping other *Shigella strains* and to *Dr F Kauffmann* Statens Seruminstitut Copenhagen for supplying 10 strains belonging to *Salmonella subgenus II*

grown on the broth base containing one per cent of lactose The same 12 strains were also lactose negative throughout an observation period of 40 days

Ten ONPG-positive strains were able to form acid from lactose within one to three days while 8 ONPG-positive strains produced acid after an incubation period of three to ten days

The 12 ONPG negative strains were next cultivated on the broth agar base containing ten per cent of lactose (35° C/22 hours), it was found that 7 of the 12 strains became ONPG positive within three hours, whereas 5 strains were persistently negative, even if the number of subcultures was increased

Klebsiella oxytoca (*Flugge* 1886), *Lautrop* 1956

Thirty three strains were all promptly ONPG positive and became lactose-positive within one or two days of incubation

Klebsiella pneumoniae

Thirty five strains belonging to this species were all rapidly ONPG positive (*i e* within 20 minutes) and formed acid from lactose within one to three days at 35° C

Klebsiella ozaenae

Only four strains were examined, they were all ONPG-positive Three were lactose positive within one to three days, while one became lactose-positive after 6 days

Klebsiella rhinoscleromatis

Nineteen strains were all both ONPG negative and lactose-negative, they were observed for 26 days for the latter reaction

Enterobacter aerogenes (*Kruse* 1896) *Edwards & Hormaeche* 1960

Five strains were ONPG positive, and lactose positive within one or two days

Enterobacter cloacae (*Jordan* 1890) *Edwards & Hormaeche* 1960

All the 30 examined strains were ONPG positive within three hours Only one strain required an incubation exceeding 20 minutes to be read as positive This particular strain did not produce acid in the lactose-containing medium within 21 days, whereas all the remaining 29 strains formed acid within one to twelve days

(In this connection "latent" means that the presence of the enzyme is not demonstrated by the standard ONPG test, and extraordinary precautions such as lowering the growth temperature or adding ten per cent of lactose to the growth media are required)

Salmonella and Arizona

Strains of this kind have all been considered as belonging to the same main group but divided into three subgroups according to *Kauffmann's* subgenera (1961)

Subgroup I—subdivided into serotypes—comprises: 10 strains of *S typhi*, 12 strains of *S paratyphi B*, 4 strains of *S paratyphi B var Java*, 15 strains of *S typhimurium*, 2 strains of *S enteritidis*, 2 strains of *S indiana*, 5 strains of *S newport*, 2 strains of *S munchen*, 4 strains of *S chicago* and one strain each of the following *S weltevreden*, *S anatum*, *S tennessee* and *S infantis*

All the 60 strains were ONPG-negative, and lactose-negative throughout 21 days of observation

It was impossible to demonstrate "latent" β-galactosidase, using the previously mentioned procedures

Subgroup II is represented by one strain from each of the following serotypes: *S humber*, *S tranoroa*, *S artis*, *S locarno*, *S basel*, *S bloemfontein*, *S merseyside*, *S rowbarton*, *S haddon*, *S jacksonville*, *S memphis* and *S schleissheim*

Five of these 12 strains were ONPG-positive within three hours, whereas the 7 remaining strains were persistently negative All the 12 strains were lactose-negative throughout a period of 21 days

Subgroup III, which is identical with *Arizona*, is represented by 38 strains, 35 of which were ONPG-positive within three hours Twenty-three were positive within 20 minutes

Twenty-five strains produced acid from lactose within 2 18 days, whereas 13 strains were lactose-negative throughout 26 days of observation

Three strains were ONPG-negative even after the lactose concentration in the growth medium and the number of subcultures on this medium were increased

These 3 strains were all lactose-negative and dulcitol-negative Two of them were malonate-negative, whereas 1 was positive According to *Kauffmann* the 3 ONPG-negative strains can be placed in subgenus II or III under the genus *Salmonella* The placing of the strains, however, depends upon how much weight is placed on the various biochemical tests

Citrobacter freundii

Of this species 30 strains were examined, 18 of which were promptly ONPG-positive, whereas 12 strains were negative if they had been

grown on the broth base containing one per cent of lactose The same 12 strains were also lactose negative throughout an observation period of 40 days

Ten ONPG positive strains were able to form acid from lactose within one to three days while 8 ONPG positive strains produced acid after an incubation period of three to ten days

The 12 ONPG negative strains were next cultivated on the broth agar base containing ten per cent of lactose (35° C/22 hours) it was found that 7 of the 12 strains became ONPG positive within three hours whereas 5 strains were persistently negative, even if the number of subcultures was increased

Klebsiella oxytoca (Flugge 1886) Lautrop 1956

Thirty three strains were all promptly ONPG positive and became lactose positive within one or two days of incubation

Klebsiella pneumoniae

Thirty five strains belonging to this species were all rapidly ONPG positive (*i e* within 20 minutes) and formed acid from lactose within one to three days at 35° C

Klebsiella o aenae

Only four strains were examined they were all ONPG positive Three were lactose positive within one to three days while one became lactose positive after 6 days

Klebsiella rhinoscleromatis

Nineteen strains were all both ONPG negative and lactose negative they were observed for 26 days for the latter reaction

Enterobacter aerogenes (Kruse 1896) Edwards & Hormaeche 1960

Five strains were ONPG positive and lactose-positive within one or two days

Enterobacter cloacae (Jordan 1890) Edwards & Hormaeche 1960

All the 30 examined strains were ONPG positive within three hours Only one strain required an incubation exceeding 20 minutes to be read as positive This particular strain did not produce acid in the lactose containing medium within 21 days whereas all the remaining 29 strains formed acid within one to twelve days

(In this connection "latent" means that the presence of the enzyme is not demonstrated by the standard ONPG test, and extraordinary precautions such as lowering the growth temperature or adding ten per cent of lactose to the growth media are required)

Salmonella and Arizona

Strains of this kind have all been considered as belonging to the same main group but divided into three subgroups according to *Kauffmann's* subgenera (1961)

Subgroup I—subdivided into serotypes—comprises 10 strains of *S typhi*, 12 strains of *S paratyphi B*, 4 strains of *S paratyphi B var Java*, 15 strains of *S typhimurium*, 2 strains of *S enteritidis*, 2 strains of *S indiana*, 5 strains of *S newport*, 2 strains of *S munchen*, 4 strains of *S chicago* and one strain each of the following. *S weltevreden*, *S anatum*, *S tennessee* and *S infantis*

All the 60 strains were ONPG-negative, and lactose-negative throughout 21 days of observation

It was impossible to demonstrate "latent" β-galactosidase, using the previously-mentioned procedures

Subgroup II is represented by one strain from each of the following serotypes: *S humber*, *S tranoroa*, *S artis*, *S locarno*, *S basel*, *S bloemfontein*, *S merseyside*, *S rowbarton*, *S haddon*, *S jacksonville*, *S memphis* and *S schleissheim*

Five of these 12 strains were ONPG-positive within three hours, whereas the 7 remaining strains were persistently negative All the 12 strains were lactose-negative throughout a period of 21 days

Subgroup III, which is identical with *Arizona*, is represented by 38 strains, 35 of which were ONPG positive within three hours Twenty-three were positive within 20 minutes

Twenty-five strains produced acid from lactose within 2–18 days, whereas 13 strains were lactose negative throughout 26 days of observation

Three strains were ONPG-negative even after the lactose concentration in the growth medium and the number of subcultures on this medium were increased

These 3 strains were all lactose-negative and dulcitol-negative Two of them were malonate-negative, whereas 1 was positive According to *Kauffmann* the 3 ONPG negative strains can be placed in subgenus II or III under the genus *Salmonella* The placing of the strains, however, depends upon how much weight is placed on the various biochemical tests

Citrobacter freundii

Of this species 30 strains were examined, 18 of which were promptly ONPG positive, whereas 12 strains were negative if they had been

Ser kielensis

Of 6 strains 4 were lactose positive within the first day whereas 2 strains were positive within four days

Ser rubidaea

This organism was described by *Stapp* in 1940 as *Bacterium rubidaeum* but in this laboratory it is classified as a *Serratia* (*Lautrop* 1962)

Eight strains were all lactose positive 6 of them formed acid within the first day of incubation and the 2 remaining strains after two weeks

Ser alba

This organism classified here as a *Serratia* (*Lautrop* 1962) is called *Aerobacter liquefaciens* by most authors

Eleven of 14 strains were unable to form acid from lactose within an observation period of 28 days The remaining 3 strains became lactose positive after 4 14 days

Proteus and Providencia

Nine strains of *P vulgaris* 6 strains of *P mirabilis* 25 strains of *P rettgeri* 21 strains of *P morganii* and 9 strains of *Providencia* were examined

All 70 strains were ONPG negative and lactose negative throughout 30 days of observation Latent" β galactosidase could not be demonstrated

Yersinia pseudotuberculosis (*Pfeiffer* 1889) *van Loghem* 1946

Fifty strains were examined and all were rapidly ONPG positive All of the strains were lactose negative throughout an observation period of 26 days

Aeromonas hydrophila

Forty eight strains were examined All of the strains were ONPG positive 44 within 20 minutes and 4 within three hours

These 4 late ONPG positive strains were lactose negative throughout a period of 26 days while 9 strains produced acid from lactose within one to three days and 35 strains did so after 3 14 days

An experiment made with one of the late ONPG positive strains showed that the reaction coud be promoted by raising the concentration of lactose in the growth media whereas the growth temperature did not seem to be of decisive importance

Hafnia alvei (Bahr 1919) Vagn Moller 1954

This series comprises 30 strains, 25 of which were ONPG positive within 20 minutes Five strains were ONPG-negative if cultivated at 35° C

Seven of the strains formed acid from lactose within one to three days, while 16 strains became lactose-positive within 3–21 days at 35° C The remaining 7 strains were unable to produce acid in a lactose medium throughout a period of 30 days, incubated at 35° C or 22° C Among these are the 5 ONPG-negative strains

Of the 5 strains that were ONPG-negative when grown at 35° C, three became positive within 20 minutes when grown at 22° C for 22 24 hours, for these three strains, the low growth temperature was an absolute requirement for the demonstration of the enzyme Cultivation at 35° C caused the ONPG test to be negative, even if media containing ten per cent of lactose were used Cultivation at 30° C resulted in an ONPG test that was positive within six to eight hours At a growth temperature of 22° C the ONPG reaction was positive in the three temperature sensitive strains, even if the broth base without lactose was used

Hafnia flava

This organism was found by *Dresel & Stickl* in 1928 and described by *Cruikshank* (1935) under the name of *Bacterium typhi flavum*, but in this laboratory it is called *Hafnia flava* (*Lautrop* 1962)

Seven strains were examined, all of them were ONPG-positive within 20 minutes Only one of these strains was lactose-positive within one day, whereas the remaining 6 strains were lactose negative for 26 days

It should be noted that the pigment of this organism may be inconvenient when one is reading the ONPG test When performing the test with such strains, one should make comparisons with a suspension of bacteria without the addition of ONPG reagent, unless it is preferred to centrifuge the test tubes before reading in which case the supernatant must be definite yellow if the reaction is to be regarded as positive

Serratia

All of the following 48 strains belonging to the genus *Serratia* were ONPG positive within three hours, 43 of them became positive within 20 minutes

Ser marcescens

Of 20 strains from this species 2 formed acid from lactose within three days, 6 strains were late-positive (3 14 days), while the remaining 12 strains were lactose-negative for 28 days

roups of Green fluorescent Pseudomonades

Egg yolk reaction	Soluble hemolysin	Flagellation	Reference strains typical for the group
0	+	monotr	Ps aeruginosa ATCC 7701 NCTC 8303
0	0	multitr	Ps ovalis ATCC 8209 ATCC 950
+	+	multitr	Ps fluorescens NCIB 3756
0	0	multitr	Ps schuylkilliensis Chiba Univ AHH 36
0	0	multitr	Ps sp PJ 7a8 Statens Seruminstitut
0	+	multitr	Ps sp PJ 846 Statens Seruminstitut

Ps ovalis (Group No 2, Table 1)

All the 64 strains investigated were both ONPG negative and lactose-negative The *Hugh & Leifson* medium containing lactose was observed for 50 days

Ps fluorescens (Group No 3, Table 1)

All the 73 strains were ONPG negative, and lactose negative throughout 50 days

Ps schuylkilliensis and other Lactose Positive Pseudomonades

Nine strains of *Ps schuylkilliensis* (Group No 4) and 13 strains of other lactose positive *Pseudomonas strains* (12 strains belonging to Group 5 and one to Group 6) were used in this investigation

All of the 22 strains were ONPG negative, whereas they all produced acid from lactose in *Hugh & Leifson*'s medium They required 6–20 days of incubation at 22° C to change the colour of the indicator

According to the observations of *Stodola & Lockwood (1947)*, it is probable that the acid formed is lactobionic acid, this would explain why there is a discrepancy between the two tests

Vibrio

Twelve strains of *Vibrio comma* and 20 strains of other *Vibrio* species were examined All the strains were part of *Heiberg*'s collection (*Heiberg* 1935)

All 32 strains were ONPG positive within 20 minutes and produced acid from lactose within 2 8 days

Pasteurella multocida (P septica)

Of 16 strains, 5 were promptly ONPG positive while the remaining 11 were ONPG negative

TABL

Some Differential Characteristics o

Group No	Growth at 37°C	$NO_3 \rightarrow NO_2 \rightarrow N_2$	*Gelatine* liq 22° C	Hugh & Leifson's O F medium Acid from						
				d arabinose	lactose	adonitol	sorbitol	mannitol	inositol	erythrito
1	+	+	+	+	0	0	0	+	0	0
2	d	0	0	0	0	0	0	0	0	×
3	0	+	+	+	0*	+	d	+	+	+
4	d	0	+	+	+	0	0	0	+	0
5	0	0	0	+	+	0	0	0	d	0
6	0	0	0	+	×	0	+	+	0	0

d = different results with various strains
× = late positive (2nd–7th day)
* = variable reaction after 2–6 weeks
+ = weak positive reaction in some strains after 2–6 weeks

Aeromonas formicans (Crawford 1954) Pivnick & Sabina 1957

All the examined 16 strains were rapidly ONPG-positive Seven of these formed acid from lactose within 1–3 days, while the remaining 9 became lactose-positive after 3–12 days

Pseudomonas

From a large material of green fluorescent pseudomonades under investigation in the Institute, 6 fairly well-characterized groups, comprising altogether 279 strains, were selected for the present investigation [1] Their differential characteristics are shown in Table 1

All the 279 strains were grown at 22° C for 40 42 hours

Ps aeruginosa (Group No 1, Table 1)

The examination comprises 120 strains, 6 of which were found to be ONPG-positive within three hours and the remaining 114 strains all ONPG-negative None of the 120 strains were able to form acid in *Hugh & Leifson's* oxidation-fermentation medium, containing one per cent of lactose, throughout a period of 50 days

It was impossible to enhance the ONPG reaction of the 6 ONPG positive strains either by raising the lactose concentration in the growth media or by changing the growth temperature to 30° C, 35° C or 42° C

Conversely, it was found, as mentioned in a previous paper, that the 6 strains were still ONPG-positive if repeatedly grown on the minimal substrate devoid of lactose

It was impossible to elicit an ONPG splitting by representatives of the ONPG-negative strains, using the previously mentioned procedures

[1] The author is very much indebted to *Dr O Jessen* Statens Seruminstitut Copenhagen, for permission to make use of his material and results

TABLE 3

Results of a Comparison between the ONPG Test and the Conventional Lactose-Fermentation Test Comprising 343 Strains from the Family Pseudomonadaceae and 90 Strains from other Families within the Gram Negative Rods

Organism	Number of strains	ONPG positive			ONPG negative		
		Acid from lactose			Acid from lactose		
		1-3 days	>3 days	none (3 weeks or more)	1-3 days	>3 days	none (3 weeks or more)
Aeromonas hydrophila	48	9	35	4			
Aeromonas formicans	16	7	9				
Pseudomonas aeruginosa	120			6			114
Pseudomonas fluorescens	73						73
Pseudomonas ovalis	64						64
Pseudomonas shuylkilliensis	9					9	
Other lactose positive Pseudomonas spp	13					13	
Vibrio comma	12	5	7				
Vibrio species	20	7	13				
Actinobacillus lignieresi	9	2	7				
Pasteurella multocida	16	5					11
Pasteurella haemolytica	2		1				1
Pasteurella ureae	13						13
Cytophaga anitrata	15				15		
Cytophaga lwoffi	3						3

Actinobacillus lignieresi [1]

Nine strains were examined. They were all promptly ONPG positive. Only 2 strains formed acid from lactose within one to three days, while the remaining 7 strains required three to eight days of incubation at 35° C to be read as positive.

Cytophaga anitrata (*Schaub & Hauber* 1948) *Lautrop* 1961

All the 15 strains examined were ONPG negative, but formed acid from lactose in *Hugh & Leifson*'s medium within one to three days. According to *Villecourt & Blachere* (1955), lactobionic acid is the probable cause of the acidification of the medium.

Cytophaga lwoffi (*Audureau* 1940) *Lautrop* 1961

Only 3 strains were examined; they were all ONPG-negative, and lactose-negative for 21 days.

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1 The thanks of the author are due to *Dr W. Frederiksen*, Statens Seruminstitut, Copenhagen, who performed the ONPG test on the strains belonging to the genera *Actinobacillus* and *Pasteurella*.

The ONPG-positive strains produced acid from lactose within one to two days, whereas the 11 ONPG-negative were lactose-negative, too, for a period of 30 days

Pasteurella haemolytica

Only 2 strains from this species were examined One was promptly ONPG-positive and lactose-positive, whereas the other was ONPG negative, and lactose-negative for 30 days

Pasteurella ureae (P haemolytica var urea, *Henriksen & Jyssum* 1961)

Thirteen strains were examined and all were ONPG-negative, and lactose-negative for 30 days

TABLE 2

Results of a Comparison between the ONPG Test and the Conventional Lactose Fermentation Test Comprising 579 Strains from the Family Enterobacteriaceae

Organism	Number of strains	ONPG positive			ONPG negative
		Acid from lactose			Lactose negative (3 weeks or more)
		1 3 days	>3 days	none (3 weeks or more)	
Klebsiella pneumoniae	36	36			
Klebsiella oxytoca	33	33			
Klebsiella ozaenae	4	3	1		
Enterobacter aerogenes	5	5			
Enterobacter cloacae	30	15	14	1	
Escherichia coli	32	28	2	2	
Escherichia dispar	9	8	1		
Serratia marcescens	20	2	6	12	
Serratia kielensis	6	4	2		
Serratia *rubidaea*	8	6	2		
Serratia alba	14		3	11	
Hafnia flava	7	1		6	
Yersinia pseudotuberculosis	50			50	
Shigella dysenteriae serotype 1	9		3	6	
Shigella sonnei	15		12		3
Salmonella arizonae (subgroup III)	38	10	12	13	3
Hafnia alvei 35° C	30	7	16	2	5
Hafnia alvei 22° C	30	7	16	5	2
Citrobacter *freundii* 1 % lac	30	10	8		12
Citrobacter freundii 10 % lac	30	10	8	7	5
Salmonella (subgroup II)	12			5	7
Klebsiella rhinoscleromatis	19				19
Escherichia alcalescens	6				6
Shigella flexneri					
Shigella boydi					
Shigella dysenteriae except *serotype* 1	36				36
Salmonella (subgroup I)	60				60
Proteus spp and Providencia	70				70

bably are devoid of permease The situation appears to be a little more doubtful if a positive ONPG reaction is combined with a late fermentation (three days or more) β galactosidase is present of course whereas the wild type cells of the bacterial strain in question can hardly be said to possess permease During growth in a lactose containing medium permease positive mutants will probably appear in the culture (*Lewis* 1934 *Lowe* 1960) in accordance with the well known mutative fermentation Another possibility is that a defective cell membrane is found in an unknown proportion of the bacterial population in this way direct contact between the substrate and a certain amount of β galactosidase could be established In either case the amount of active β galactosidase in a certain suspension of cells is probably small and the ONPG reaction correspondingly slower than it is when β galactosidase is found in the majority of cells

It can be seen from the results that certain aerobic organisms such as *Cytophaga anitrata* and certain *Pseudomonas* strains produce acid from lactose if examined in *Hugh & Leifson* s media even though β ga

v aldehyde group of lactose to a carboxylic group This would explain the discrepancy observed between the two tests

Attention should be drawn to the quite unexpected finding that 6 out of 120 strains of *Pseudomonas aeruginosa* were able to split ONPG Provided that the cell membrane of these organisms is permeable for lactose the β galactosidase should probably be characterized as constitutive since the velocity of the ONPG splitting was independent of the presence of lactose in the growth medium

In the identification of Gram negative rods the ONPG test appears to have certain practical advantages over the ordinary lactose fermentation test This is most easily understood by considering how an identification is usually performed The ordinary process is to compare the reaction pattern of the unknown strain with the corresponding patterns of the most common bacteria recorded in a diagnostic table If the representatives of a species or a group behave uniformly in a particular test it is possible to record a clear cut + or — in the table whereas much variation among the individual strains necessitates the use of another symbol e g *d* for different results or *v* for variable results It is quite obvious that a pattern mainly consisting of + or — results is more definite than one containing a number of *d* results and that the first pattern consequently allows unknown strains to be identified with greater confidence

Now it can be seen from Table 2 that if the results of the lactose fermentation test are replaced by the results of the ONPG test in the diagnostic tables a number of *d* results can be supplanted by + results and if the tables are made on the basis of the results obtainable within

DISCUSSION

For many years, fermentation media containing carbohydrate and an indicator have demonstrated their usefulness in diagnostic bacteriology It is, however, a well-known fact that a positive "fermentation test" only means that during growth enough acid has been formed to change the colour of the indicator Generally, it is not possible from a positive "fermentation test" to establish conclusively that the bacteria concerned possess one particular enzyme, because a positive "fermentation test" nearly always presupposes the presence of a number of enzymes In contrast to the "fermentation test", the method of "direct" enzyme-demonstration by a chromogenic enzyme substrate has the advantage that it can show *whether* or not a particular enzyme (*i e* the one which splits the chromogenic substrate) is present

In the present investigation the direct demonstration of β galactosidase and the lactose-fermentation test have been compared, and it could be established that the results of the two tests do not always agree If both tests are performed, the following combinations of results are possible.

ONPG	+	+	—	—
lac	+	—	+	—

Of these combinations, the second and the third obviously deserve special consideration in an evaluation of the usefulness of the ONPG test in diagnostic bacteriology

As the ONPG test is often able to reveal a β-galactosidase which the *lactose-fermentation test cannot bring to light, it may be concluded* that the ONPG test is much more sensitive than the fermentation test As a clear-cut example, *Yersinia pseudotuberculosis* might be mentioned, all 50 strains were promptly ONPG-positive, but persistently lactose-negative

The difference in the sensitivity of the two methods is probably due in most cases to a difference in the permeability of the cell membrane for lactose and ONPG

It is more doubtful whether the difference might be explained by assuming that cell autolysis with subsequent liberation of enzymes is greater in the heavy suspensions than in the bacterial population in the fermention tube If this were the case it would mean that the easier demonstration of the enzyme by the ONPG test was only a matter of quantity

If both tests *are performed one should, therefore, often* be able to say whether or not galactoside-permease is present in the bacteria examined

A positive ONPG reaction and a fast positive lactose reaction (appearing within three days) undoubtedly mean that both β-galactosidase and permease are present A positive ONPG test and a negative lactose reaction signify that β-galactosidase is present, whereas the cells pro-

SUMMARY

A material of 579 strains belonging to the family *Enterobacteriaceae*, 343 to the family *Pseudomonadaceae* and 90 strains to other families of the Gram negative rods was compared as to the ability to split ONPG and the ability to form acid from lactose

It is concluded that the ONPG test is much more sensitive than the ordinary lactose-fermentation test for demonstrating β galactosidase in bacteria and that it is possible by the use of both tests to distinguish between two types of fermentation results

It is recommended that the ONPG test should be included among the routine tests used in clinical bacteriology

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48 hours—a forced practice in clinical bacteriology—the improvement is still more striking

Although it is probable that an investigation of a greater number of strains will disclose minor deviations from the findings reported here, it is not probable that they will be so frequent as to call for a radical revision of the present conclusions *Szturm-Rubinsten* (1962) and *Szturm-Rubinsten & Piechaud* (1963) have investigated large numbers of *Alcalescens-Dispar* strains and of *Shigella* strains, and the differences between their results and the present ones are only what one might expect when the sizes of the two materials are considered

The ONPG reaction is also remarkable for its rapidity, permitting a final reading to be made after 3 hours

A special advantage over the conventional methods is the reliability of early negative readings, which with great probability excludes the presence of the *β-galactosidase*

If the answer "late lactose fermentation" is still considered to be of diagnostic importance, a supplementary ONPG test will often tell whether this answer is to be expected

If all important biochemical tests used in bacteriological routine identification could be performed according to the same principles as the ONPG test, a significant improvement in the practical identification of bacteria would probably be made Besides, it is probable that a change from the conventional diagnostic methods to the proposed "direct" enzyme-demonstrating ones might be economical because of the smaller amount of media required

CONCLUSION

The results of the two tests are not identical In the first place, the ONPG test is much more sensitive for demonstrating β-galactosidase than the lactose-fermentation test, consequently it is more often positive than the fermentation test In the second place, the fermentation tube reveals acid production in some cases where the ONPG test is negative, because some bacteria oxidize lactose to lactobionic acid instead of splitting the β-galactoside bond Consequently the ONPG test cannot simply replace the lactose-fermentation tube Its value lies in the fact that it contributes information which makes *possible in most* cases an early prediction of the result of the fermentation test, and in some cases a tentative prediction of the process underlying the acid production in the fermentation tube Therefore, the maximum information is obtained by using both tests, but it seems that if only one is to be used the ONPG test is the more useful—at least for medical bacteriologists, who so frequently have to deal with members of the family *Enterobacteriaceae*

A COMPARISON OF HIGH AIR PRESSURE TOLERANCE AND RADIATION RESISTANCE IN E. COLI B

By

ANNA STINA MALMBORG

Received 18 ıx 63

According to the "target theory" of *Lea* (1946) the lethal effect of radiation results from chemical damage to some vital structure, a "target", in the cell The damage might be caused by oxidation products of water irradiated in the presence of oxygen (*Alper & Howard Flanders* 1956 *Howard Flanders & Alper* 1957, *Howard Flanders & Moore* 1958) This could for instance explain the reduction in radiosensitivity seen under anaerobic conditions (*Hollaender, Stapleton & Martin* 1951, *Hollaender & Stapleton* 1953) *Lea* also mentions that the decomposition products of proteins or other cell constituents produced as a result of irradiation may act as cell poisons in quite low concentration *Dale* (1940, 1942) emphasizes that the inactivation of enzymes may be important

Witkin (1947) has discussed the resistance of E coli B r to irradiation as possibly beeing due to its greater efficiency in either the repair of a compound necessary for division, or in the rapid destruction or neutralization of a cell poison

As previously shown (*Heden & Malmborg* 1961) the aerat[illegible]

[illegible] appearance of free radicals Against this background it was considered to be of interest to investigate if some correlation exists between increased radiation resistance and resistance to high partial pressures of oxygen

MATERIAL AND METHODS

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su[illegible] irradiation and the fraction of [illegible] bacteria was tested at intervals This procedure was first performed at

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Witkin (1947) has discussed the resistance of E coli B/r to irradiation as possibly beeing due to its greater efficiency in either the repair of a compound necessary for division, or in the rapid destruction or neutralization of a cell poison

As previously shown (*Heden & Malmborg* 1961) the aeration under pressure above 5 kg cm² causes a growth inhibition of E coli B The effect was discussed in terms of the formation of an oxygen dependent inhibitor or the appearance of free radicals Against this background it was considered to be of interest to investigate if some correlation exists between increased radiation resistance and resistance to high partial pressures of oxygen

MATERIAL AND METHODS

Two strains of E. coli were compared the UV sensitive E coli Bc obtained from E coli B by *Cohen* (1959) and the UV resistant mutant E coli Bc/r *isolated by* double UV irradiation by *Bertani* according to the method of *Witkin* (1950)[1] The two strains were simultaneously subjected to UV irradiation and the fraction of surviving bacteria was tested at intervals This procedure was first performed at

This investigation was supported by a grant from the *National Cancer Institute* (C-5799) *U.S. Public Health Service*

[1] The strains were kindly provided by *Dr G Bertani* Karolinska Institutet Stockholm

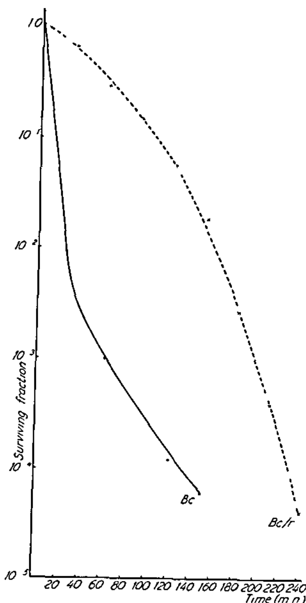


Fig 1
Sensitivity of E coli Bc and Bc/r to UV irradiation

the outset of the experimental series and a second time when the whole series was finished As shown in Fig 1 there was a marked difference in UV sensitivity between the two strains This difference was the same at the outset and after completion of the experimental series

The aeration apparatus was the same as previously described (*Hedén & Malmborg* 1961) Pulsaerator tubes (*Hedén* 1957 *Hedén & Malmborg* 1958) each containing 5 ml of Difco broth were placed in a pressure vessel A two way valve intermittently provided access from the vessel to the open air and to compressed air from a gas cylinder

Experiments at different pressures were performed independently For each pressure 6 pulsaerator tubes of each strain were inoculated with 0 5 ml of a suspension in 10 ml saline of the growth from 2 agar slopes incubated for 18 hours at 37° C

Determinations of growth were performed after 3 and 6 hours Three tubes of each strain aerated under increased pressure and one control tube aerated at atmos

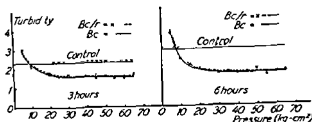


Fig 2 A

Growth of E coli Bc and Bc/r under aeration at various pressure levels (measured turbidimetrically)

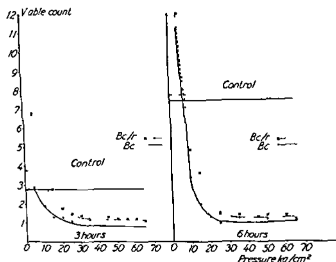


Fig 2 B

Growth of E coli Bc and Bc/r under aeration at various pressure levels (measured by viable count)

pheric pressure were removed The growth was measured both turbidimetrically and by viable count according to the soft agar method (*Adams* 1959) When preparing the curves showing the relation between pressure and viable count or turbidity Fig 2 A and B) each recorded value is divided by the value at the outset of each experiment so that the figures represent the number of times the original value has increased The average of all control readings at normal air pressure is represented as a horisontal line

RESULTS

The strain E coli Bc and the strain E coli Bc/r showed no great difference in growth intensity *i e* no increased resistance to pressure could be observed with the mutant E coli Bc/r compared to E coli Bc Fig 2 A and B) In the graphs E coli Bc/r may seem a little more resistant but this might be explained by the fact that the strain grew better than E coli Bc under all the pressures used and in the controls The data so far available permit no detailed analysis of the graphs for

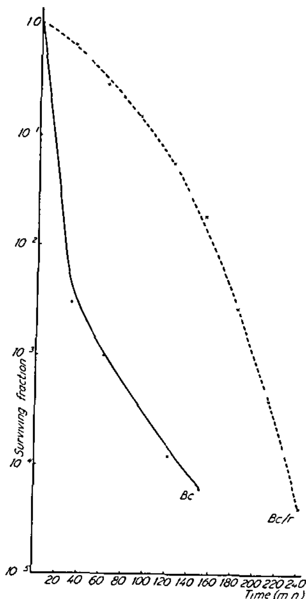


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The sensitivity to radiation and the sensitivity to H_2O_2 are well correlated This indicates that H_2O_2 is an intermediate in radiation damage (*Engel & Adler* 1961, *Adler* 1962) H_2O_2 also seems to be produced under high gas pressure (*Luck* 1954), when the same type of free radicals may be produced as in water exposed to radiation

A radiation resistant strain has some ability to neutralize the toxic products formed from free radicals through a chemical protective agent, or by employing metabolic pathways, which allow the cell to repair or bypass radiation-caused damage (*Witkin* 1947, *Adler & Engel* 1961) An increase in catalase activity has been observed in bacteria grown under pressure (*Lück* 1954) It is of interest to note, however, that the power to produce this enzyme can hardly be a major difference between radiation sensitive and resistant cells since there is no conspicuous correlation between catalase content and radiation sensitivity (*Adler* 1962)

If the oxygen tension is sufficiently increased, the cellular defense against radiation damage will be overruled and a toxic effect of oxygen is revealed (*Gilbert et al* 1957) The radiation resistant strain reported in the present experiments is obviously unable to repair the damage caused by high air pressure as efficiently as it repairs the damage caused by irradiation In this respect it is almost as deficient as the radiation sensitive strain

SUMMARY

An UV-sensitive and UV resistant strain of E coli B were cultured under various air pressures The fraction of bacteria surviving pressure was higher for the UV-resistant, than for the UV-sensitive strain However, the difference between the fractions was not so large as between the fractions surviving UV-treatment This shows that the factors responsible for the cell damage under conditions of irradiation and under elevated air pressures are not identical

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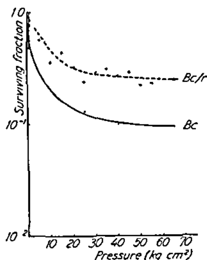


Fig 3 A

Surviving fractions of 3 hours old cultures of E coli Bc and Bc/r after pressure treatment Vertical lines represent deviation in 13 determinations ($\pm\sigma$)

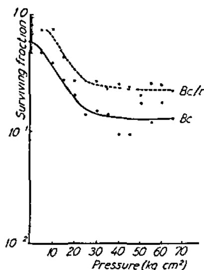


Fig 3 B

Surviving fractions of 6 hours old cultures of E coli Bc and Bc/r after pressure treatment Vertical lines represent deviation in 13 determinations ($\pm\sigma$)

instance of the increased bacterial counts at 30 kg/cm² in the case of E coli Bc (Fig 2 B) The reduction in turbidity relative to the control at pressures between 10 and 65 kg (Fig 2 A) is *probably due to the* autolysis of bacteria

The fraction of bacteria surviving pressure treatment is higher for the UV-resistant than for the UV-sensitive strain (Fig 3 A and B), but the difference is not of the same order of magnitude as the difference between the fractions of the same strains surviving UV-treatment (Fig 1) as earlier mentioned This indicates that different factors damage the cells under UV irradiation and under high pressure It would be of interest in later experiments to study the effect of various gas pressures on the growth of E coli B under anaerobic conditions

DISCUSSION

If radiation damage to bacterial cells, and the growth inhibition by elevated air pressure, were mediated by the same chemical mechanism, one might assume that the development of radiation resistance is caused by a mechanism which would cause resistance also to high air pressure The fact that the effects of radiation may be caused by oxidation products of water (*Alper & Howard-Flanders* 1956, 1957, *Howard Flanders & Moore* 1958), together with the fact that the toxic effect of high air pressure appears essentially to be a function of its oxygen content (*Heden & Malmborg* 1961), *would seem to make such a correlation a* reasonable hypothesis However, the preceding section indicates that there is no correlation between radiation resistance and resistance to high air pressure in E coli B

FROM THE MUNICIPAL VIROLOGICAL LABORATORY AND THE VIROLOGICAL LABORATORY OF THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF GOTHENBURG SWEDEN

STUDIES ON ANTIGEN—ANTIBODY SYSTEMS WITH REFERENCE TO MONONUCLEOSIS INFECTIOSA

By

ÖRJAN STRANNEGÅRD and ERIK LYCKE

Received 07 viii 63

In 1932 *Paul & Bunnell* reported the discovery that patients with mononucleosis infectiosa (M I) developed antibodies agglutinating sheep erythrocytes Since then the so called Paul Bunnel test (P -B test) has been widely used for the diagnosis of M I Later *Bailey & Raffel* (1935) found that sera from patients with M I contained haemolysins directed against sheep and ox erythrocytes On the basis of these findings the ox cell haemolysin test (O C H test) has been worked out (*Mason* 1951)

Stuart et al (1936) demonstrated a thermostable heterogenetic antigen in ox erythrocytes that reacted with sera obtained from patients with M I This so called "mononucleosis antigen" was also found in sheep erythrocytes although at a lower concentration The antigen has been studied *i a* by *Tomcsik & Schwarzweiss* (1947, 1948)

Leyton (1952) and *Eyquem et al* (1955) observed that ox cell haemolysins *could not* be absorbed by sheep erythrocytes and concluded that ox cell haemolysins and sheep cell agglutinins are different antibodies Their results bring up the question of whether or not there is more than one heterogenetic antigen determinant on ox and sheep erythrocytes responsible for the reactions with M I sera Studies of the antigen antibody systems related to M I *are* presented in the present report, including results obtained by means of diffusion-in-gel methods

MATERIAL AND METHODS

Antigenic preparations Beef or sheep erythrocytes were washed three times with physiological saline autoclaved at 110° C for 20 m[illegible] sand for one hour A double volume of 1 per[illegible] and the preparation was left overnight in the [illegible] was pipetted off filtered through gauze [illegible] and concentrated by pervaporation in dialysis tubing at room temperature The concentrated preparation was then dialyzed overnight against physiological buffered

The skilful technical assistance of Miss *Margreth Peterson* is greatly appreciated

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recorded The plates were
washed for some days in physiological buffered saline pH 7 2 and then dried and stained with amido black

Immunoelectrophoretic technique The comparative immunoelectrophoretic technique, an elaboration of the method of *Grabar & Williams* (1953), introduced by *Walsworth & Hanson* (1960), was used The experiments were performed in 1 per cent agar prepared with veronal Na buffer The agar was poured on a 8 × 8 cm glass plate to which two 8 × 2 cm glass plates were attached with adhesive tape at opposite sides The two smaller glass pieces were permitted to hang down into the buffer vessels which were filled with 0 025 molar veronal Na buffer, pH 8 2 The electrophoretic separation was performed at 5 V/cm for 70 min At the completion of the run precipitation analyses were performed by cutting longitudinal basins in the agar an filling these basins with reactants The plates were then kept in a humid atmosphere for at least 3 days then washed for 3 days in physiological saline and finally dried and stained with amido black

EXPERIMENTAL

The Relationship between the Ox Cell Haemolysin Titres and Sheep Cell Agglutinin Titres

O C H and P -B tests were performed on 2,989 sera obtained from patients with suspected M I during the years 1959–1961 Serum dilutions ranging from 1/16–1/1024 were used The sera given no reaction in dilution 1 16 were considered as negative

As shown by Fig 1 a linear relationship was obtained between the titres of the two tests Almost 10 times higher titres were obtained with the O C H test than were found employing the P -B test The fact that no sera were tested above the dilution 1/1024 in the tests contributes to the levelling off of the curve

Reactivity of Sera after Absorption with Ox and Sheep Erythrocytes

Sera with high titres in the O C H , O C A and P -B tests were absorbed with an equal volume of packed native sheep erythrocytes and retested for the presence of haemolysins and agglutinins The results are given in Table 1 The mean titres of 9 sera tested before and after the absorption are listed

The sheep cell agglutinin titres (P -B) were decreased about 100 times by the absorption whereas the ox cell agglutinin titres (O C A) and the ox cell haemolysin titres (O C H) were lowered about 7 and 8 times, respectively Absorptions with sheep erythrocytes were repeated twice and then the sheep cell agglutinins were no longer demonstrable The O C H and O C A titres, however, were unchanged by these absorptions

Absorption with native, heated or trypsinized ox erythrocytes reduced the concentration of the agglutinins and the haemolysins to a degree where they could not be demonstrated

saline at pH 7 2 The *final yield* of antigenic preparation from 1 35 l packed erythrocytes had a volume of about 65 ml *The ox and* the sheep cell preparations obtained in this way are in the following designated a_{01} and a_S *respectively*

A portion of the sheep cell preparation was further concentrated by pervaporation almost to dryness and then dialyzed *against buffered* saline Another portion of the preparation a_S was given a pH of 2 2 by *adding 0 1 N HCl* Preparation then occurred and after centrifugation the sediment was resuspended in a small quantity of buffered NaCl at pH 7 2

The ox cell preparation a_{01} was found to give unspecific fuzzy precipitates with normal human serum *and a portion of* this preparation was therefore absorbed with human serum known to be negative in P B and O C H tests This preparation concentrated 5 times *in dialysis tubing* was used as a reference antigen and is referred to as a_{02}

A method similar to that described by *Schwartzweiss & Tomcsik* (1948) for the extraction of mononucleosis antigen was used *i e* treatment with 80 per cent *boiling ethyl alcohol* Extraction of autoclaved beef or sheep erythrocytes which had been stored with 1 per cent *carbolic acid* was performed but the use of concentrated beef stroma was not attempted

Attempts were also made to dissolve precipitating antigens of heated ox cells by treatment with 0 1 N NaOH

Antisera Specimens of human sera sent to the virological laboratory for diagnostic purpose were used The specimens were stored at —30° C. *Before being used in* the experiments to be described the sera were thawed and *then stored in the* refrigator They were tested in O C H and P B tests before use in the particular experiments Of the sera which were *positive in the P B* test only those were used which were found to be specific after absorption according to *Davidsohn* (See below)

The human serum (1314/62) used as the reference antiserum was obtained from a 13 year old patient with the clinical picture of mononucleosis infection including icterus and exanthema and a differential white blood cell count strongly suggestive of this disease This serum had an O C H titer of 16 384 and a P B *titre of* 1 024 Anti human globulin sera were obtained from Behringwerke

Paul Bunnell test Sera were titrated in two fold dilution *steps To* 0 5 *ml of* serum dilution was added 0 5 ml of a 2 per cent suspension of washed *sheep erythrocytes* and 1 ml of veronal Na buffer pH 7 0 The tubes were placed in the *refrigerator* overnight The readings were made the next day after an additional incubation at 37° C for two hours The titres were defined as reciprocals of the *serum dilution in* the last tube with agglutination of the sheep cells

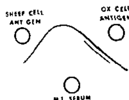
O C H test The ox cell haemolysin test was performed according to *Petersen et al* (1956) Serum dilutions were made as described before To 0 5 ml of each serum dilution was added 0 5 ml of a 1 per cent suspension of washed *ox erythrocytes* 0 5 ml 15 per cent guinea pig serum and 0 5 ml veronal Na buffer The tubes were kept in a waterbath at 37° C for 15 min and then read The titres were defined as reciprocals of the final serum dilution in the last tube with haemolysis

Only inactivated sera were used in the P B and O C H tests

Absorption tests There were carried out by allowing antigen and antibody to react for one hour at room temperature After centrifugation at 3000 rpm for 15 min the supernatant was pipetted off an tested for the presence of antibodies Routine absorption tests according to *Davidsohn* (1937) were performed as follows 0 25 ml of serum was absorbed with 1 25 ml of a 10 per cent emulsion of heated guinea pig kidney or ox erythrocytes

Ox cell agglutination test (O C A) The test was carried out as described by *Tomcsik* (1960) One ml of packed washed ox erythrocytes was mixed with 19 ml of a trypsin solution at a concentration of 1 10 000 After centrifugation the packed erythrocytes were resuspended in physiological saline to a 1 per cent suspension 0 1 ml serum was absorbed for one hour with 0 3 ml guinea pig kidney emulsion and after centrifugation 0 1 ml of the supernatant was titrated in two step dilutions Of the trypsinized ox erythrocyte suspension 0 2 ml was added to 0 1 ml of serum dilution The tubes were allowed to stand at room temperature for 10 min and then centrifuged at 3 000 rpm for 3 min The agglutination was assessed after tapping on the tubes

Double diffusion in gel technique The Ouchterlony technique in the micromodification introduced by *Walsworth* (1957) was used The experiments were per



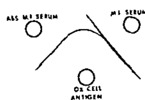


Fig 2 Fig 3

Fig 2 *Comparative double diffusion analysis of antigenic preparations from sheep erythrocytes and ox erythrocytes* Diagrammatic representation of the precipitation pattern observed when antigens were tested against the reference antiserum (1314/62) The sheep cell preparation as and the ox cell preparation a_0 were used

Fig 3 *Comparative double diffusion analysis of an unabsorbed M.I serum and the same serum absorbed with sheep erythrocytes* Diagrammatic representation of the precipitation pattern observed when a M I serum (399 63) and the same serum absorbed 3 times with equal volumes of packed sheep cells were compared by means of an ox cell antigen (a_{02}) Before absorption the serum had an O C.H titre of 4096 and a P B titre of 512 and after absorption the O C.H titre was 2048 and the P B titre less than 8

precipitation lines The antigen a_S showed precipitating activity only if it was concentrated about 20 times by pervaporating or by precipitation at pH 2 2

In a few instances sheep cell preparations gave rise to one line which identified with the major line obtained with the ox cell preparations In Fig 2 is shown the precipitation pattern observed when a sheep cell (a_S) and a ox cell (a_{02}) antigen preparation were compared by means of the reference serum The ox cell antigen formed two lines, one of which interfered with the line formed by the sheep cell antigen This would indicate that there exists an antigenic factor common to ox and sheep erythrocytes which is precipitated by components in the M I serum

Absorption of the M I sera with guinea pig kidney emulsion did not diminish their precipitating activity After repeated absorption of sera with equal volumes of packed sheep erythrocytes usually no ox cell precipitins were demonstrated However, when sera having high O C.H titres after absorption with sheep cells, were analyzed, a faint precipitation line was obtained in some instances One serum (399 63) absorbed 3 times with sheep cells and then concentrated about 5 times by pervaporation was found to give rise to a precipitation line which interfered with the line obtained with the unabsorbed serum, giving partial coalescence (*Fig 3*) The precipitate observed when the unabsorbed serum was analyzed did not split into two lines when the concentrations of antigen or antiserum were changed

The antigenic preparations were tested for their ability to absorb agglutinins and haemolysins according to the method described by *Tomcsik & Schwarzweiss* (1947) It was found that the most potent precipitating ox cell antigen absorbed sheep cell agglutinins as well as

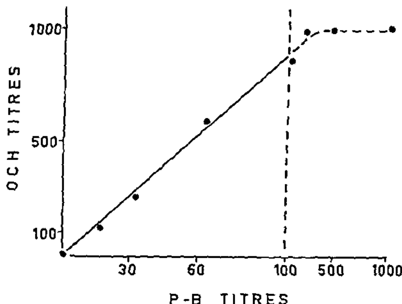


Fig 1

The relationship between O C H and P B titres Mean titre values of 2 989 sera are plotted *The two types of tests were performed simultaneously on each serum specimen Serum dilutions ranging from 1/16 to 1/1024* were used in the tests

TABLE 1

Sheep Cell Agglutinin (P B) Ox Cell Agglutinin (O C A) and Haemolysin (O C H) Titres of Sera from Patients with Mononucleosis infectiosa before and after Absorption with Native Sheep Erythrocytes Mean Values of Tests Performed on 9 Sera

Test	Mean titres	
	Unabsorbed sera	Absorbed sera
P-B	427	4
O C A	693	103
O C H	3072	356

Experiments with the Double Diffusion-in-Gel Technique

Sera with high titres in the O C H *and* P-B tests were analyzed with antigen preparations *obtained from* ox and sheep erythrocytes When precipitation occurred one well *defined line* appeared, usually after two days When ox cell preparations were used *frequently* a second days This line

precipitating ox cell antigens the one employing mechanical treatment of the heated erythrocytes and subsequent concentration was *found* superior to the other preparative *procedures attempted*

A quite reactive antigen (a_{o}) giving reproducible *results* was prepared in this way *It* was *difficult* to achieve preparations with precipitating activity from sheep erythrocytes Some *preparations* from sheep cells were, however, *obtained* which gave reproducible but faint

precipitins were, on the other hand, readily demonstrable In accord with these results the reference serum having a P-B titre of 1024 was found to precipitate at a highest dilution of 1 in 16 against antigen a_{0}.

Immunoelectrophoretic Experiments

The antigen a_{0} from ox erythrocytes and sera with high precipitating activity were used When the method of *Grabar & Williams* (1953) was employed no precipitation lines appeared With the "comparative method of *Wadsworth & Hanson* (1960), however, the ox cell precipitins could be demonstrated

As can be seen in Fig 4 the ox cell precipitins had a β-globulin mobility The precipitation arc formed when the separated reference serum reacted with the antigen a_{0} had the same localization as the β_{M} globulin precipitate of human serum The electrophoretic mobility of the ox cell antigen could not be accurately determined with the methods used

The Effect of Absorption with Anti-Human Globulin Sera

The double diffusion-in gel technique with five basin arrangement was used in such a manner that it permitted absorption of certain antibodies present in the reference serum Three basins were half-filled with saline, undiluted anti β_{2M}- or anti-γ-globulin, respectively Six hours later the reference serum was added to each of the three basins and tested for the presence of precipitating antibodies against ox cell antigens by filling the remaining two basins with either the preparation a_{01} or a_{0}.

Anti β_{2M}-globulin inhibited precipitation completely while anti γ-globulin serum did not affect the precipitation and showed no influence on the localization or the appearance of the precipitation lines To determine the specificity of the anti globulin sera they were tested immunoelectrophoretically with whole serum as antigen It was found that the anti β_{2M}- and the anti-γ-globulin sera reacted strongly and specifically with human β_{2M}- and γ-globulins, respectively

DISCUSSION

The sho[illegible]
cell h[illegible]
labora[illegible] of mononucleosis infect[illegible]
[illegible] are different antibodies or if one type of antibody is functioning in the two reactions (*Leyton* 1952, *Eyquem* 195[illegible] *Ericsson* 1960)

ox cell haemolysins to a high degree whereas precipitating sheep cell antigen absorbed the former but not the latter type of antibodies (Table 2)

TABLE 2

Inhibition of Ox Cell Haemolysins and Sheep Cell Agglutinins by Ox and Sheep Cell Precipitating Antigens

Absorbing antigen	Reciprocals of the highest antigen dilution inhibiting 4 units of	
	Ox cell haemolysins	Sheep cell agglutinins
Ox cell antigen (a_{02})	4096	512
Sheep cell antigen (a_S)	<16	256

In the table are indicated reciprocals of the highest dilution of antigen of which 0 5 ml absorbed 4 haemolysing of agglutinating units of serum pool The pool was made of 5 sera from patients with M I and had a P-B titre of 256 and a O C H titre of 4096

The specificity of the reacting precipitins in regard to M I was investigated by determining the relationship between presence of heterophilic precipitins and P-B and O C H titres in 74 sera The antigen a_{02} was used and the reference serum 1314/62 was included in all of these tests The results are listed in Tables 3 and 4

TABLE 3

The Relationship between the Occurrence of Precipitins and the P-B Titres Seventy-four Sera Were Tested with an Ox cell Preparation for the Presence of Precipitins

P B test titre	< 16	32-64	128-256	≧ 512
Precipitins demonstrated	0	1	14	16
No precipitins	22	9	12	0
Total	22	10	26	16

TABLE 4

The Relationship between the Occurrence of Precipitins and the O C H Titres Seventy four Sera Were Tested for the Presence of Precipitins with an Ox Cell Preparation

O C H test titre	< 256	512	1024
Precipitins demonstrated	0	2	29
No precipitins	29	8	6
Total	29	10	35

Precipitins could not be demonstrated in sera with O C H titres below 512 or in sera with P-B titres less than 32 In 29 out of 35 sera with O C H titres of 1024 and in all sera with P-B titres of 512 or more,

precipitins were on the other hand readily demonstrable In accord with these results the reference serum having a P B titre of 1024 was found to precipitate at a highest dilution of 1 in 16 against antigen a_0

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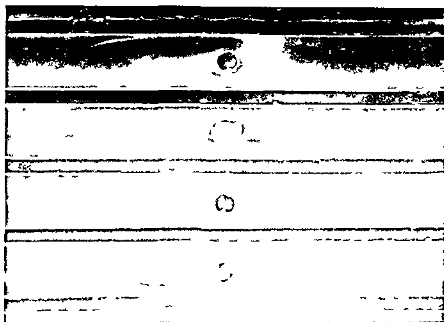
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The sheep cell agglutinin test according to *Pa* [illegible] e ox the [illegible]

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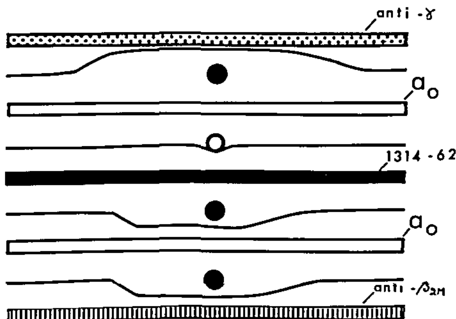


Fig 4

... mobility of heterophilic antibodies precipitating or cell antigen
representation (4b) of an immunoelectrophore
γ and β_{2M} globulins were determined by means
er part of the figure) Circular basins indicate
Filled circles reference M I serum (1314/62)
(a_{02}) In the diagrammatic representation are
s near the circular basins which are artefacts
the conclusions drawn in the context

Tomcsik & Schwarzweiss (1947) suggested the presence of a mononucleosis antigen common to ox and sheep erythrocytes. The results reported by previous investigators however do not exclude the possibility that there are different antigenic factors in ox and sheep erythrocytes reacting with sera from patients with mononucleosis.

The absorption studies of *Davidsohn* (1937) demonstrate that ox erythrocytes are able to react with the sheep cell agglutinins. Thus these agglutinins correspond to one antigenic factor common to ox and sheep erythrocytes or the agglutinins have two reactive sites one of which corresponds to an ox cell factor and the other to a sheep cell antigen. According to *Eyquem et al.* (1955) ox cell haemolysins are not absorbed by sheep erythrocytes. *Leyton* (1952) has observed however that it seems as if a very slight absorption does occur. In the present study absorption of M I sera with sheep erythrocytes reduced the concentration of ox cell haemolysins 8 to 9 times. The titres could not be further diminished by repeated absorptions indicating that the ox cells have an antigenic factor reacting with haemolysins which is not present in sheep erythrocytes. The reduction in haemolysins found after the sheep cell absorptions indicates that a part of the haemolysins are able to react with ox cell as well as sheep cell antigens.

The good correlations between P B and O C H titres may suggest that one type of antibody is reacting in the two tests. However the results of the absorption studies show that this cannot be the case but that there is one haemolytic antibody corresponding to an antigenic factor of the ox cell and another antibody reacting with antigenic factors of both ox and sheep cells given agglutination of sheep cells. The existence of the latter type of antibody may thus explain the decrease in ox cell haemolysin titres when sera are absorbed with sheep cells. Another explanation would be that there exists a third type of antibody with two different reactive sites. The ox cell agglutinins seem to be the same type of antibodies as the ox cell haemolysins as they behave similarly in the absorption tests.

A correlation between the occurrence of precipitins demonstrated by means of the double diffusion in gel technique and the occurrence of haemolysins and agglutinins could be demonstrated in M I sera. It seems reasonable to assume that the precipitins demonstrable are related to M I as are the particular haemolysins and agglutinins discussed. The most potent of the precipitating ox cell antigen preparations were found to absorb agglutinins and haemolysins to a very high extent.

The results of the diffusion in gel experiments indicate that there exists an antigen common to ox and sheep cells. This finding is in accord with the results of the absorption experiments. It is most plausible that the antigen capable of absorbing the sheep cell agglutinins is the one that gives the most distinct line in the precipitation experiments. The minor additional line observed when the ox cell antigen reacts with M I serum may correspond to the antigen antibody system

specific for the ox cell haemolysis and agglutination The minor line could not be demonstrated with certain sera in spite of their high O C H titres after absorption with sheep cells This can be explained if it is assumed that the antigenic factors demonstrated are similar as to their diffusion velocities and this is true also for the two types of antibodies It is then possible to get superimposed lines which cannot be separated by means of dilution procedures The difference between the precipitation spectra obtained with the sera 1314/62 and 399 63 (Figs 2 and 3) when analyzed by means of antigen a_0, can thus be explained, as the relative concentrations of the two types of antibodies in these sera were different The O C H titres were for serum 1314/62 16,384 before and 512 after absorption with sheep cells and for serum 399 63 4,096 and 2,048 respectively

The types of precipitation reactions observed are not those that would be expected if the antigenic factors of ox cells are determinants on the same antigenic molecule

Summing up, the results of the precipitation as well as the absorption experiments indicate that in sera from patients with M I there are at least two types of antibodies, one reacting with an antigenic factor of ox cells and the other reacting with an antigenic factor common to ox and sheep cells

The absorptions and immunoelectrophoretic experiments indicated that the antibodies precipitating ox cell antigens were β_{2M} globulins It is known that many human isoagglutinins and other antibodies directed against erythrocytes are substances of high molecular weight *Fudenberg & Kunkel* (1957) have studied heterophilic antibodies in human serum and found them all to be in the high molecular weight class Furthermore, results obtained by *Strannegård & Hanson* (in press) indicate that sheep cell agglutinins as well as ox cell haemolysins have the nature of β_{2M} globulins

SUMMARY

Heterophilic antibodies occurring in sera from patients *with mononucleosis* infectiosa and their corresponding antigens in sheep and ox erythrocytes were studied The ox cell haemolysin (O C H) titres were found to be well correlated to titres obtained in sheep cell agglutination tests (P -B tests) The ox cell agglutinins seemed to be identical with the ox cell haemolysins Absorption studies indicated that at least two types of antibodies are reacting in the O C H and P -B tests

Antigenic preparations obtained from heated and mechanically disintegrated ox and sheep erythrocytes gave rise to precipitation lines when allowed to react with sera with high O C H and P -B titres The occurrence of precipitins was found to be correlated to the concentrations of ox cell haemolysins and sheep cell agglutinins The diffusion-in gel studies demonstrated two *antigenic factors in ox erythrocytes*

One of these factors was related to the only one demonstrated in sheep erythrocytes. These findings were supported by absorption studies.

The antibodies precipitating ox cell antigens had $\beta_2 M$ globulin mobility.

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FROM THE MICROBIOLOGICAL DIVISION, THE ROYAL PHARMACEUTICAL INSTITUTE, STOCKHOLM, SWEDEN

COMPARISON BETWEEN SOME PENICILLIN-G RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS PREPARED IN VITRO AND THEIR PARENT STRAINS

2 *Immunological Studies*

By

LENNART WAHLSTROM and BIRGITTA NORKRANS

Received 7 viii 63

By means of double-diffusion agar technique, evidence was found of a change in the antigenic pattern when penicillin-G sensitive clinical strains of Staphylococcus aureus acquired penicillin-G resistance *in vitro* (*Norkrans & Bertrandsson* 1962, *Norkrans & Wahlstrom* 1962)

In the present paper, an account is given of further studies on the subject, extended to include a number of international strains and a penicillinase-producing one, as well as several antisera Press disruption has been used in addition to ultrasonic disintegration for the preparation of diffusible antigens

MATERIAL AND METHODS

Test Organisms and their Characteristics

Seven coagulase positive strains of S aureus were studied From the parent penicillin G sensitive strains (S) penicillin G resistant strains (R) were prepared as previously described (*Norkrans & Wahlstrom* 1962) resulting in R strains with penicillin G resistance capacities ranging from 40 to 6000 µg/ml (see Table 1)

As a rule penicillin resistant strains induced *in vitro* do not produce penicillinase (cf *Wallmark* 1953) in contrast to the naturally occurring penicillin resistant strains Strain 5 however was picked up from a colony of a clinical penicillin sen ... surrounded ... of a peni ... ence of the ... penicillinase production all of them were tested for penicillinase activity according to *Kornlein & Koch* (1954) ... for some strains the method was modified by usi[ng] ... [br]aine in liquid medium instead of solid medium (... [w]as necessitated by the fact that staphylococci w... [St]*einman* 1961) were used in the present investigation, whereas ... [t]ested Bacillus cereus and Escheri... penicillinase As could be expected strain ... others were not

... was made with the aid of phage typing The phages were obtained from the [Mic]robiological Associates Inc Washington D C and typing was performed according to their description (adapted from *Blair*

TABLE 1

Some Characteristics of Penicillin G Sensitive Strains (S) of Staphylococcus aureus and their Corresponding Resistant Strains (R) and Antisera Produced

Strain	Penicillin resistance capacity μg/ml	Penicillinase producer	Phage patterns: Total lysis	Phage patterns: Partial lysis (approx. 50 plaques)	Phage patterns: Some lysis (5-50 plaques)	Antisera against: Heat killed bacteria	Antisera against: Formalin killed bacteria
1 S				52A/80	52/79		A_1 S_F
R	2 000						A_1 R_F
2 S			3B/3C/3A 55/71	3A	7/29/42E/47		
R	4 000						
3 S			1C/7/47/54/75/83	42E/53/70/77	42B/81		
R	40		3B/3C/55/71/52A	1/7			
4 S						A_4 S_H	A_4 S_F
R	6 000					A_4 R_H	A_4 R_F
5 S			1/80	42B/47C/52A/54	6/44A/47/52/53/70/73/77		A_5 S_F
R	6 000		(*No growth*)				A_5 R_F
6 S							
R	4 000						
7 S							
R	1 000	—					

The strains 1, 2 and 3 S were obtained from the National Collection of Type Cultures, London, the type collection nos. are 8530, 8531, and 8532, corresponding to Cowan's types I, II and III, respectively.

The strains 4, 5, 6, and 7 S were kindly supplied by Dr. S. G. *Andrén*, the Hospital for Infectious Diseases, Norrkoping, Sweden. The clinical nos., used by us in previous papers, for 4, 6, and 7 are 1429, 339, and S 169, respectively. 5 was derived from a strain 252, as described below. S 169 was isolated from faeces, the other three from pus from middle ear.

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As a rule penicillin resistant strains induced *in vitro* do not produce penicillinase (cf *Wallmark* 1953) in contrast to the naturally occurring penicillin resistant strains Strain 5, however, was picked up from a colony of a clinical penicillin sensitive strain designated as 252 grown on a penicillin medium which was surrounded by a faint halo of bacterial growth which indicated it to be a colony of a penicillinase producing mutant (*Sybalski* 1953) In order to obtain evidence of the anticipated differences among the resistant strains with respect to penicillinase production all of them were tested for penicillinase activity according to *Kornlein & Koch* (1954) To improve the growth conditions for some strains the method was modified by using a suspension of penicillin procaine in liquid medium instead of solid medium (*Kirby* 1945) This modification was necessitated by the fact that [illegible] penicillinase (*Steinman* 1961) were used in the staph [illegible] heri presen [illegible] rain chia c [illegible] 5 was [illegible] ing

An [illegible] ton The phages were obtained [illegible] and typing was performed according to their description (adapted from *Blair* D C, [illegible]

ger 1957, *Cooper* 1956) indicate that cell wall synthesis is the target for penicillin action and since thin cell walls are produced under the influence of penicillin (*Murray et al* 1959) the reason for the higher protein release in the resistant strains as compared to that in the parent strains after exposure times exceeding 15 minutes may be a less robust cell wall of the former Thus, an exposure time of 15 minutes seemed to be optimal, even if longer times gave higher protein contents of the antigen solution and, possibly, a more pronounced antigenic pattern

TABLE 2
Numbers of Precipitation Lines in the Diffusion Plates

Antigen preparation	Antisera							
	A_1S_F	A_4S_F	A_4S_H	A_5S_F	A_1R_F	A_4R_F	A_4R_H	A_5R_F
1 S	4	1			2	2		
2 S	4	1	0		3	2	0	
3 S	1	0	0		0	2	1	
4 S		[5]*	[4]*	1		[2]*	[0]*	1
5 S				4		4	1	4
6 S		3	0			1	0	
7 S		0	0			2	0	
1 R	1	1			7	1		
2 R	3	0	0		2	2	2	
3 R	2	0	0		3	5	2	
4 R		[0]*	[2]*	4		[6]*	[3]*	4
5 R				4		4	1	4
6 R		0	0			1	0	
7 R		0	0			2	0	

* Numerals within brackets are obtained from a previous investigation (*Norkrans & Wahlstrom* 1962)

A viability test related to a turbidimetric one, showed 98 per cent mortality after 15 minutes' exposure (cf *Gale & Folkes* 1955) After this time the turbidity had decreased by 20 per cent, and after 1 hour an almost clear solution was obtained The effects of ultrasonic disintegration on the bacterial cell have been further discussed in a previous paper (*Norkrans & Wahlstrom* 1962) The suggestion of *Cohen et al* (1958) that ultrasonic disintegration may give rise to degradation products of the [illegible]
of such short d[illegible]
appeared after [illegible] believed to be a less drastic method for obtaining diffusible antigens (cf *Oeding* 1960, *Salton* 1960) It was not found however, to be of any definite advantage, and all results given below refer to experiments with ultrasonically prepared antigens for diffusion tests We have confined ourselves to recording the number of precipitation lines without subjecting them to further chemical analysis Bearing in mind the variability in immune response of the different experimental animals, differences in the antigenic

& *Carr* 1953, *Williams & Anderson* 1956, *Zierdl* 1959) Three of the strains, i e 4 6 and 7, were non typable The remaining strains had the phage patterns given in Table 1 As can be seen from this table, the induced penicillin resistant strains showed no sensitivity to phages in agreement with earlier reports (*Wallmark* 1954) An exception was strain 3, which had a phage pattern differing from that of the parent strain (cf *Fouace* 1953) This strain had, however, very low resistance capacity in comparison with the others

Certain observations were made in connection with induction of penicillin resistance The mucoid strain, 7, isolated from faeces, was found to contain rods when the penicillin concentration of the medium was raised This was initially ascribed to contamination, even though the technique used gave no grounds for such a postulation However, since the phenomenon was consistently seen in a large number of duplicate experiments, and the coccal forms reappeared after several transfers at the same penicillin level, it was later interpreted as the "rod phenomenon previously reported by *Gale* (1948) It was not observed in other strains used in this investigation In comparison with other strains, 7 had a considerably higher protein content after ultrasonic disintegration Loss of pigmentation was also observable during the increase in resistance capacity, especially in the deep yellow strain 3 (cf *Wallmark* 1953)

Antigen, Antisera and Diffusion Test

Antigen for diffusion tests were prepared by

A Ultrasonic disintegration The procedure has been described in detail in a previous paper (*Norkrans & Wahlstrom* 1962)

B Press disruption of antigens according to *Hughes* (1951) and *Edebo* (1960) Lyophilized bacteria (0 200 g) were suspended in 2 ml of sterile citrate phosphate buffer of pH 7 4 and cooled in an ice water bath The cold suspension was placed in the press and pre cooled overnight on solid CO_2 to get the desired temperature throughout the material This was then pressed as quickly as possible with the aid of an hydraulic jack The pressed material was diluted with 8 ml of buffer allowed to attain equilibrium in an ice water bath for 2 hours and then centrifuged at 13000 g for 20 minutes Merthiolate 1 10 000 was added to the antigen solutions

The preparation of antisera and of antigens for immunization and the procedures for the immunizations ring tests and agar diffusion tests are described elsewhere (*Norkrans & Wahlstrom* 1962)

RESULTS

The investigation was designed to compare the antigenic patterns of the sensitive strains (S) with those of the corresponding resistant ones (R), and to demonstrate possible changes when resistance had been acquired It was of the greatest importance therefore to avoid as far as possible laboratory procedures which could have modifying effects on the antigens of sensitive and resistant strains, especially if the modifications were different in the different strains

The crude antigen solutions obtained after ultrasonic disintegration were tested for protein content (*Lowry et al* 1951) in order to determine the disintegration effect in the sensitive and the corresponding resistant strains After 15 minutes' exposure, the protein release was about the same in 4 S and 4 R, i e 2050 and 1650 μg ml respectively After prolonged exposure, it increased more rapidly in the resistant strains For example, 30 minutes treatment gave a protein value of 2720 μg/ml in strain 4 S and 4950 μg ml in 4 R The corresponding values after 60 minutes' exposure were 4740 and 6510 μg/ml

Since numerous investigations (e g *Lederberg* 1957, *Park & Stromin-*

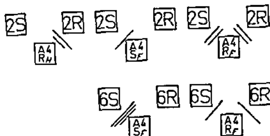


Fig 3

Precipitation patterns of strains 2 and 6 against antisera from strain 4

2 S and 6 S = antigen of sensitive strains 2 and 6 respectively

$A4S_F$ = antiserum against formalin killed sensitive strain 4

2 R and 6 R = antigen of resistant strains 2 and 6 respectively

$A4R_F$ = antiserum against formalin killed resistant strain 4

$A4R_H$ = antiserum against heat killed resistant strain 4

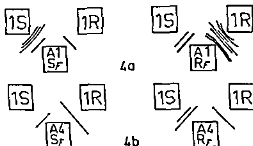


Fig 4

Precipitation patterns of strain 1 against homologous sera and heterologous sera (strain 4)

1S = antigen of sensitive strain 1

$A1S_F$ = antiserum against formalin killed sensitive strain 1

1R = antigen of resistant strain 1

$A1R_F$ = antiserum against formalin killed resistant strain 1

$A4S_F$ = antiserum against formalin killed sensitive strain 4

$A4R_F$ = antiserum against formalin killed resistant strain 4

highly dominant protein bands probably correlated to the mucoid appearance of 7 These disturbing proteins were removed by fractionation in the usual way with ammonium sulphate

As in the experiments with strain 3 (Fig 1) precipitation lines were obtained only with antiserum to 4R in this case only with A_4R_F no precipitation lines being detected with A_4S_H or A_4S_F

The above treated S and R strains usually caused a different number of
Th
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Su ... have three precipitation lines against A_4S_F whereas 6R gave none (see Fig 3)

Reaction of antisera of strain 1 against homologous and heterologous

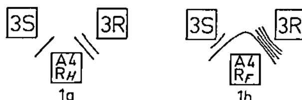


Fig 1

Precipitation patterns of strain 3 against antisera from strain 4
3S = antigen of sensitive strain 3 A4R_H = antiserum against heat-killed resistant strain 4
3R = antigen of resistant strain 3 A4R_F = antiserum against formalin killed resistant strain 4

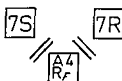


Fig 2

Precipitation patterns of strain 7 against antisera from strain 4
7S = antigen of sensitive strain 7
7R = antigen of resistant strain 7 A4R_F = antiserum against formalin-killed resistant strain 4

patterns of one or two lines have not been considered as "distinct differences", even if the use of pooled antisera gives a certain guarantee for correction of this source of error

Antisera against Heat-Killed and Formalin-Killed Bacteria

The results are summarised in Table 2 It is evident that A_4R_F gave more precipitation lines against antigen solutions of all the S and R strains tested than did A_4R_H in corresponding tests (cf Figs 1, 2 and 3) Similar results were obtained when A_4S_F and A_4S_H were compared in analogous tests Thus, since antisera against heat-killed bacteria exhibited less striking precipitation patterns than did those against *formalin-killed* bacteria, the latter only were prepared for the remaining strains *i e* 1S, 1R, 5S and 5R

Antisera against Sensitive and Resistant Strains

Reaction of antisera of strain 4 against heterologous strains Table 2 shows that A_4R_F gave more precipitation lines against antigen solutions of all the heterologous S and R strains tested (except 6S) than did A_4S_F in corresponding tests Furthermore, A_4S_H did not cause any precipitation reactions with antigen solutions from any strains, whereas A_4R_H did This richer precipitation pattern of the antisera prepared against 4R is demonstrated for some of the strains in Figs 1 3

As regards strain 7 (Fig 2) it will be noticed that it initially gave no precipitation lines with A_4S_H, A_4S_F, or A_4R_H and A_4R_F Electrophoresis, performed concurrently on the disintegrated cell material, revealed two

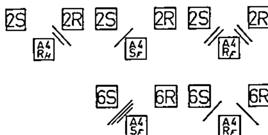


Fig 3

Precipitation patterns of strains 2 and 6 against antisera from strain 4

2 S and 6 S — antigen of sensitive strains 2 and 6 respectively

$A4S_F$ = antiserum against formalin killed sensitive strain 4

2 R and 6 R = antigen of resistant strains 2 and 6 respectively

$A4R_F$ = antiserum against formalin killed resistant strain 4

$A4R_H$ = antiserum against heat killed resistant strain 4

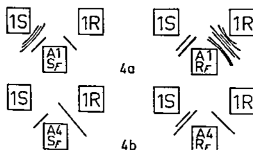


Fig 4

Precipitation patterns of strain 1 against homologous sera and heterologous sera (strain 4)

1S = antigen of sensitive strain 1

$A1S_F$ = antiserum against formalin killed sensitive strain 1

1R = antigen of resistant strain 1

$A1R_F$ = antiserum against formalin killed resistant strain 1

$A4S_F$ = antiserum against formalin killed sensitive strain 4

$A4R_F$ = antiserum against formalin killed resistant strain 4

highly dominant protein bands probably correlated to the mucoid appearance of 7 These disturbing proteins were removed by fractionation in the usual way with ammonium sulphate

As in the experiments with strain 3 (Fig 1) precipitation lines were obtained only with antiserum to 4R in this case only with A_4R_F no precipitation lines being detected with A_4S_H or A_4S_F

The above treated S and R strains usually caused a different number of precipitation lines in reactions against one and the same antiserum This is most striking in the case of strains 3 and 6 3R caused five precipitation lines with A_4R_F whereas 3S gave only two lines (see Fig 1b) Similarly 6S gave three precipitation lines against A_4S_F whereas 6R gave none (see Fig 3)

Reaction of antisera of strain 1 against homologous and heterologous

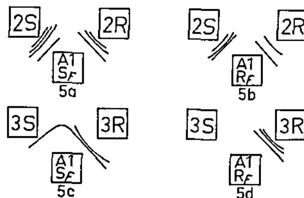


Fig 5

Precipitation patterns of strain 2 and 3 against antisera from strain 1
2 S and 3 S = antigen of sensitive strains 2 and 3 respectively
$A1S_F$ = antiserum against formalin killed sensitive strain 1
2 R and 3 R = antigen of resistant strains 2 and 3 respectively
$A1R_F$ = antiserum against formalin killed resistant strain 1

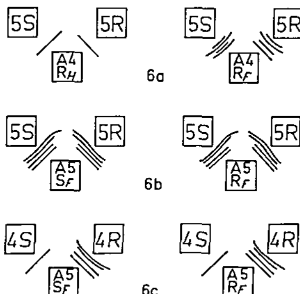


Fig 6

Precipitation patterns of strain 5 against heterologous sera (strain 4 Fig 6a) and homologous sera (Fig 6b) and the precipitation patterns of strain 4 against heterologous sera (strain 5 Fig 6c)
4 S and 5 S = antigen of sensitive strains 4 and 5 respectively
4 R and 5 R = antigen of resistant strain 4 and 5 respectively
$A4R_H$ = antiserum against heat killed resistant strain 4
$A4R_F$ = antiserum against formalin killed resistant strain 4
$A5S_F$ = antiserum against formalin killed sensitive strain 5
$A5R_F$ = antiserum against formalin killed resistant strain 5

strains Results similar to those described above were obtained in tests with A_1S_F and A_1R_F against heterologous strains 3R reacted with A_1R_F with three precipitation lines whereas 3S gave none (Fig 5d) although the 3S antigen preparation showed shared antigen with 3R in reactions with A_1S_F (Fig 5c)

In test with homologous strains, however, more distinct differences were obtained 1R gave a rich precipitation pattern when tested against A_1R_F but only one precipitation line against A_1S_F, whereas 1S reacted with two lines with A_1R_F and 4 lines with A_1S_F (Fig 4)

Reaction of antisera of strain 5 against homologous and heterologous strains Strain 5 differed from all other strains tested in that 5S and 5R showed the same number of precipitation lines in tests against both the homologous antisera A_5S_F and A_5R_F (see Fig 6b) and the heterologous antisera A_1R_H and A_1R_F (see Fig 6a) Futhermore, both A_5S_F and A_5R_F gave the same precipitation pattern with 4S and 4R (see Fig 6c)

As in previous experiments (*Norkrans & Wahlstrom* 1962, cf Table 2), however, a distinct difference was apparent between 4S and 4R, in reactions with A_5S_F and A_5R_F, 4S gave only one precipitation line, whereas 4R gave four such lines

DISCUSSION

Previous experiments (*Norkrans & Wahlstrom* 1962) with antisera produced from strain 4 (there designated 1429) against homologous sensitive and resistant strains indicated that a difference did, in fact, exist betw

pattern of the sensitive strains when they acquire penicillin resistance, further antisera were produced In addition, several heterologous strains were tested against these antisera in order to investigate if they give rise to one or more antigens common to all the penicillin-induced re sistant strains

In the experiments with strain 1 against homologous sera (Fig 4a), distinct differences in the antigenic pattern of the sensitive and the resistant strains were in fact found

In analogous experiments with heterologous strains, the diffusion tests gave less distinct differences between the sensitive and resistant strains and were sometimes lacking altogether

From the above mentioned investigations it is known that the cell wall is more thin in penicillin G resistant staphylococci than in penicil lin G sensitive ones For this reason it is easier to prepare rich antigen solutions from the resistant than from the sens

the differences in the antigenic patterns obtained be tween the S and R strains

However, differences like those found here in tests with A_1S_F and A_1R_F against homologous strains where, on the one hand, 1R gave seven precipitation lines with A_1R_F and only one with A_1S_F whereas, on the other hand, 1S gave four precipitation lines with A_1S_F and two with

A_1R_F, cannot be fully explained in that way The results seem rather to indicate intrinsic differences in the antigenic structure of sensitive and resistant strains These results are quite in accordance with results previously obtained for strain 4 (see Table 2), where 4S gave five precipitation lines in A_4S_F whereas 4R gave none, and 4S gave two precipitation lines in A_4R_F whereas the homologous 4R precipitated six lines

Thus, it is apparent that if the antigenic differences between a penicillin sensitive strain and a corresponding penicillin-induced resistant one are to be clearly visualized, the corresponding antiserum against each of them must be prepared

The complex antigenic structure of staphylococci with frequently occurring cross reactions, and the scanty knowledge of the mechanism of *in vitro* induced penicillin resistance complicate the interpretations of the results

It is, however, apparent that the different resistant strains have no "resistance antigen" in common, which could be possible only if the resistance in all cases had been acquired by the same mechanism, directly reflected in the antigenic pattern Different mechanisms are known, however, and a change in the antigenic pattern may be a secondary *effect resulting in formation of separate strain-specific antigens*

This last possibility is indicated by Fig 4a which shows seven precipitation lines between 1R and A_1R_F, but only one between the same strain and A_1R_I (Fig 4b)

This may be valid for all induced penicillin-G resistants which acquire their resistance in other ways than by producing penicillinase This seems to be confirmed in tests with strain 5—the penicillinase producer In this case, antigen preparations from the sensitive strain gave the same precipitation pattern with all four antisera tested as did the resistant strain (Fig 6a, 6b) On the other hand, the two antisera A_5S_F and A_5R_F gave the same precipitation pattern when tested against 4 R and 4 S (Fig 6c) Thus, Fig 6 does not reveal any differences between 5 S and 5 R, but confirms the difference between 4 S and 4 R, as has been shown earlier with homologous sera *(Norkrans & Wahlstrom 1962)*

Strains 5 S and 5 R derive from a non-penicillinase producing strain 252 S, from which a non penicillinase producing resistant 252 R was produced This strain, on the other hand, differed from its parent sensitive strain with respect to antigenic pattern (*Norkrans & Bertrandsson* 1962) Thus, in this case *it has been possible to show that penicillin-G resistant strains developed from one and the same strain produced in different ways*, as penicillinase producer and non penicillinase producer— differ in their antigenic behaviour *i e* no differences exist between a penicillinase producing resistant strain and its parent strain, whereas the antigenic pattern of a penicillin-G resistant strain, with resistance acquired in another way, differs from that of the cor

responding penicillin G sensitive strain *Stern & Elek* (1957) stated that agglutination tests disclosed no change in the antigenic structure of *Staphylococcus aureus after* it had become penicillin resistant It is possible that they used penicillinase producers, although they did not mention the fact

SUMMARY

1 Comparisons have been made between the antigenic pattern of seven coagulase positive resistant strains of Staphylococcus aureus, and that of their corresponding parent strain They were tested against eight antisera representing sensitive and resistant strains Both formalin killed and heat killed bacteria were used for immunization
2 The strains have been phage typed, only one showed a deviating phage pattern after acquiring penicillin resistance
3 On acquirement of penicillin resistance, differences in the antigenic pattern were observed in strains that were not penicillinase producing
4 One strain 5 was isolated as a penicillinase producer Even after acquiring a markedly high degree of penicillin resistance it did not present differences in the antigenic pattern similar to those of the non penicillinase producing strains

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DEPARTMENTS OF GENERAL PATHOLOGY AND ANATOMY, THE ROYAL DENTAL COLLEGE UNIVERSITY INSTITUTE OF GENERAL PATHOLOGY, AND RIGSHOSPITALET, MEDICAL DEPARTMENT A COPENHAGEN

BACTERIA IN WHIPPLE'S DISEASE

I Results of Cultivation from Repeated Jejunal Biopsies Prior to, During, and After Effective Antibiotic Treatment

By

N. Kok, R. Dybkær and J. Rostgaard

Received 19.viii.63

Whipple's disease is well named because of the unsurpassed description of the syndrome given by *Whipple* (1907).

Since then, more than a hundred cases have been verified. Still, an explanatory designation is not possible, because a definite proof of the aetiology is lacking.

The advent of the intestinal biopsy capsule now permits the taking of *in vivo* biopsies of jejunal tissue. During the last few years light and electron microscopical examinations of such specimens have revealed several cases of Whipple's disease, and an earlier diagnosis has led to effective antibiotic treatment. Furthermore, it has been established beyond doubt that whenever the jejunal lamina propria from an untreated patient with Whipple's disease is examined by electron microscopy it contains rod shaped bacteria in macrophages and extracellularly (*Chears Jr. & Ashworth* 1961, *Yardley & Hendrix* 1961, *Kurtz, Davis Jr. & Ruffin* 1962, *Caroli, Stralin & Julien* 1963d, *Rostgaard* 1964).

As no reports of successful cultivation of pathogenic, rod shaped microorganisms from diseased tissue could be found it was decided to make an attempt at isolating bacteria from jejunal biopsies—in spite of the expected difficulties with the unavoidable contamination by the luminal microflora.

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& Julien …
their th…
… as well as electron microscopical and microbiological studies the importance of the bacteria in the tissue was

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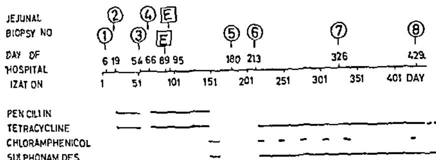


Fig 1

The chronological relation between jejunal biopsies and therapy in a case of Whipple s disease

The patient s first day at Rigshospitalet med dept A was 12 III 1962 during the recorded period of 439 days he was hospitalized five times Encircled numbers refer to consecutive jejunal biopsies the letter E in a square designates extraction of teeth Penicillin was given as phenoximethylpenicillinnatrium NFN 600 mg daily the dose of tetracycline chloride NFN was 1 g daily and of chloramphenicol NFN 1 g daily the sulphonamide medication was sulphacombin NFN 4 g daily for 14 days and later sulphamethoxipyridazine NFN 500 mg daily

was reinstituted with tetracycline and a sulphonamide (sulphamethoxipyridazine NFN) augmented for short periods by chloramphenicol During this period of 225 days a seventh (326th day) and an eighth (429th day) biopsy were taken and since the 439th day the treatment (apart from prednisone) consists in sulphonamide with

... electrophoretic pattern still points to an immune response

MATERIALS AND METHODS

Per ... ... ile (1957) ... ys (Rigs ... The cap ... the small piece of mucosa liberated and thereafter handled gently and with sterile instruments exclusively The biopsy was cut into five or six pieces One or two were immediately fixed for histological examination and—from the second biopsy onwards—four were assayed for viable bacteria Each of these four specimens was washed thoroughly in three ...

Blood broth peptone broth containing 1 10 volume of horse blood

Blood ascites agar peptone broth containing 1 10 volume of horse blood 1/20 volume of ascitic fluid and agar (15 g l)

"Chocolate" ascites agar as blood ascites agar except that the blood was added to the substrate at 80° C.

stressed anew; after having isolated a strain of *Corynebacterium anaerobium* from an inguinal lymph node in a patient with Whipple's disease they concluded that this anaerobic or microaerophilic bacterium is the aetiological agent of the disease.

REPORT OF CASE

In order to assess the results it is necessary to relate the jejunal biopsies to the condition of the patient and the treatment he received (cf. Fig 1). A detailed case report will be published elsewhere.

J H, a Danish, male farm worker, was born 3 III 1928 Since 1954 he had been suffering from attacks of migrating, afebrile polyarthritis and since 1959, additionally, from periodical, abdominal oppression and loose bowels January 1961 he was hospitalized on account of weight loss; the patient was suspected of a cancer of the stomach, but at laparotomy only enlarged mesenteric lymph nodes were found, histological examination led to the diagnosis: Whipple's disease In spite of infusions of blood and fat, and peroral prednisone administration, his condition deteriorated, and continued weight loss eventually necessitated hospitalization at Rigshospitalet, medical department A, 12 III 1962 (case no 18576)

(In the following, consecutive days are reckoned from the first day in Rigshospitalet, including several periods of increasing length spent at home)

The patient presented a typical picture of Whipple's disease with emaciation (body weight 42 5 kg, height 1 65 m), palpable, peripheral lymph nodes, blood pressure 110/80 mm Hg, erythrocyte sedimentation rate 25 mm in 1 h, anaemia (blood haemoglobin concentration (as iron) 6 4 mmol/l = 107 g/l) with a low mean erythrocyte volume, subnormal serum concentrations of iron, total iron binding capacity, protein (57 g/l), albumin (39 g/l), calcium, and cholesterol, normal concentrations of sodium, potassium, and chloride, an elevated concentration of seromucoid (296 mg/l); the urine contained an augmented concentration of an electrophoretic fract-

is demonstrated, blood

(fatty acids excretion

absorption of xylose

The lupus erythematosus phenomenon in the blood was positive and immunoelectro-

an immune response The small intestine showed

ad been continued nearly without interruptions,

(the technique is described below), taken on the

ie diagnosis and is fully described later in this

Rostgaard (1964)

The second biopsy, on the eighteenth day was *again* examined histologically, and several kinds of bacteria were isolated by cultivation (see later) Treatment with penicillin (phenoximethylpenicillinnatrium NFN) and tetracycline chloride NFN was then given for 33 days (cf Fig 1) and supplemented by fat and protein hydrolysate intravenously The patient responded by gaining in body weight, blood haemoglobin, and serum protein concentrations

The third biopsy (54th day) and the fourth (66th day) bracketed a period without antibiotics, which were then resumed as before during a period of 82 days followed by fourteen days of chloramphenicol and a sulphonamide (sulphacombin NFN)

The patient reached a normal body weight and haemoglobin concentration in the blood, the xylose absorption had become normal but the faecal excretion of fat and nitrogen remained elevated

Due to extensive deep caries and parodontitis most of the patient's teeth were extracted on the 89th and 95th days (at Rigshospitalet Dental Department) The pulpae as well as the teeth themselves were cultivated for microorganisms

The fifth jejunal biopsy (180th day) and the sixth (213 day) were taken 19 and 52 days, respectively, *after* discontinuation of the antibacterial treatment Suspected microorganisms could still be isolated from the fifth and consequently, therapy

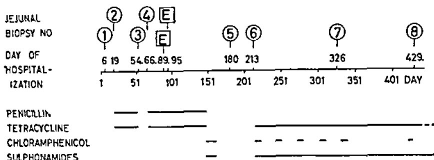


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... immunoelectrophoretic pattern still points to an immune response

MATERIALS AND METHODS

Peroral ... ile (1957)
was ... ys (Rigs-
hospi ... The cap
sule ... the small piece of mucosa liberated and
thereafter handled gently and with sterile instr...

... and then squeezed between pincers

Choice of substrates The difficulty in isolating microorganisms from a jejunal biopsy lies of course in the unavoidable ... intestine The bacteria in the ... of the influence ... more they ... quiring wit ...

Blood broth peptone broth containing 1/10 volume of horse blood

Blood-ascites agar peptone broth containing 1/10 volume of horse blood 1/20 volume of ascitic fluid and agar (15 g l)

"Chocolate" ascites agar as blood ascites agar except that the blood was added to the substrate at 80° C

Semi solid agar peptone broth containing agar 1 5 g/l

Serum broth peptone broth with 1/10 volume of horse serum

Peptone water with peptone 10 g/l in diluted meat extract broth and with the addition of a specific substance for fermentative assay

Primary incubation One squeezed tissue specimen was streaked on a blood ascites agar plate and the rest left at the end of the trail another specimen was transferred to blood broth These two cultures were incubated aerobically at 37° C. The two remaining specimens were treated likewise but incubated anaerobically (in Seitz jars) Finally, the pincers used for squeezing were dipped in serum broth for aerobic incubation and then left with the points in semi fluid agar which was incubated anaerobically

Secondary isolation procedures The surfaces of the *aerobic plates* were examined daily material from one or from each of several colonies of each macroscopically distinct kind was taken for microscopy of a Gram stained slide and for a new aerobic streak plate culture on blood agar and sometimes other appropriate media The *anaerobic plates* were examined in a similar manner after 72 or 96 hours and subcultivated anaerobically as well as aerobically Material from the *aerobic blood broth* was taken following 6 18 24 48 and 72 hours of incubation and each time streaked on five chocolate ascites agar plates and five blood agar plates for aerobic isolation of colonies which were treated as mentioned for the aerobic plates By the second third and fourth biopsies material was also streaked after 96 and 120 hours incubation but this procedure was abandoned as new strains did not appear The subcultures after 6 and 18 hours never yielded bacteria not found in one of the later subcultures The *anaerobic blood broth* was likewise subcultivated anaerobically at 72 144 and sometimes at 216 or 240 hours of age

By continued use of selective and enrichment media most of the detected strains were obtained in pure cultures as shown by colonial appearance and microscopy

Identification was attempted on morphological and biochemical criteria when a pure culture had been achieved

All strains were examined in gelatin and semi solid agar stabs for liquefaction and mobility respectively the production of indole nitrite from nitrate hydrogen sulphide and catalase was tested as well as the effect on lithmus milk presumed *Lactobacilli* were assayed for aciduricity *Corynebacteria* for polar staining and *Nocardia* or *Streptomycetes* for acid fastness

All not strictly anaerobic strains were tested in the usual way for effect on each of the following substances contained in peptone water or serum peptone water at a concentration of 10 g/l aesculin L arabinose arginine dextrin D fructose D galactose D glucose glycerol hippurate (with glucose) inulin lactose maltose mannitol raffinose L rhamnose saccharose salicin D sorbitol trehalose urea (with glucose) and D xylose strains from the three first biopsies were tested for production of acids the remaining strains also for production of gas Streptococcal strains were classified according to the Sherman criteria

Blood samples for aerobic and anaerobic cultivations were drawn by venous puncture after the skin had been swabbed twice with iodine in ethanol the blood was taken directly into two flasks containing peptone broth making a blood concentration of 1/10 (v/v)

Special considerations In consequence of the electron microscopical findings on the first and second jejunal biopsies as well as a study of pertinent literature our efforts were mainly directed towards examination of small rodlike bacteria whereas the cocci obvious *Lactobacilli* and large rods were not identified as species

The taxonomy follows Bergey s Manual of Determinative Bacteriology 7th ed 1957 as closely as possible

Light microscopical examination for bacteria in sections (5 6 µm thick) of paraffin embedded tissue was made after methylene blue staining and differentiation with acetic acid 2 g/l Gram staining using differentiation with ethanol to a varying degree and counter staining with neutral red was performed and in addition the method of *Brown & Brenn* (1931) for Gram staining was used

Electron microscopy of tissue embedded in Vestopal W was made as described by *Rostgaard* (1964)

Extracted teeth The substrates were chosen according to the reasons given in the section Peroral jejunal biopsies Each of the *upper teeth* was crushed with a sterile pair of forceps and with a sterile scalpel some dental pulpa was scraped off and added to two tubes with *serum ascites* broth the dental fragments were divided

between two further tubes with the same substrate and one from each pair incubated aerobically the other anaerobically both at 37° C. Subcultures from the aerobic cultures were made at 6 24 and 48 hours after the primary incubation each time on five blood agar plates and on five 'chocolate' agar plates Likewise the anaerobic subcultures were made after 24 and 48 hours

The *lower teeth* were treated in a similar manner except for flaming of the surface before crushing This procedure may have reduced the external flora

RESULTS

Cultivation

As might be expected a large number of different microorganisms and strains could be isolated from the various biopsies Nearly all were obtained in pure culture and classified as closely as was considered necessary or possible The first biopsy was not investigated for viable microorganisms For the second one the technique used was not quite as reliable as for the rest The results from the second biopsy, therefore, are not considered fully representative

Several small, rod shaped microorganisms—Gram positive as well as Gram negative—were isolated from the biopsies These microbes are summarized in Table 1, which thus lists the strains that might appear in the lamina propria as small, thin rods.

Furthermore the following strains were characterized Several species of *Micrococcaceae* in biopsies nos 2, 3, 4, 5, 6, 7, and 8 Several different strains of *Staphylococcus aureus* in nos 2, 3, and 7 *Neisseria flava* in no 6, *N perflava* in no 7, and *N catarrhalis* & *sicca* in no 8 Presumed or proved *Veillonellae* were found in all biopsies (nos 2 through 8) The identification of these strains was sometimes uncertain, because the cultures often died before purification was effected Several strains of *Streptococci* of the viridans- and enterococcus [illegible] were [illegible] 4, [illegible] in [illegible] of *Lactobacilli* were found in nos 3 through 8 Some rather large *Bacilli* (0 9 × 4 μm) were present in no 3 and some rather large, Gram negative rods (0 7 × 3–7 μm) together with some Gram negative rods (0 7 × 6–7 μm) in chains appeared in no 5 and nos [illegible] *[illegible]tridia* were observed in biopsy no 7, [illegible] In biopsy no 8 two kinds of short, [illegible]e seen (0 5–0 7 × 0 7–1 1 μm) *Candida albicans*[1] was encountered in biopsies nos 2, 3, 4, 7, and 8 Finally, several kinds of threadlike, Gram negative rods (0 5–1 μm in diameter)—some possibly *Leptotrichiae*—occurred in nos 3, 6, and 7

The more pertinent strains (cf Table 1) may in summary be described as follows

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[1] Dr Per Holm M D., Statens Seruminstitut (Mycological Department) is gratefully thanked for confirming the identification of the species

TABLE 1

The Most Important Strains of Rodlike Bacteria Isolated from Jejunal Biopsies and Extracted Teeth For Relation to Antibacterial Treatment, Cf Fig 1

Bacterial strain no	taxonomy	Date day of hospitalization serial no	Jejunal biopsies							Extracted teeth	
			30 III 62	4 V 62	16 V 62	7 IX 62	9 X 62	31 I 63	14 V 63	8 VI 62	14 VI 62
			19	54	66	180	213	326	423	85	9[illegible]
			2	3	4	5	6	7	8	maxill	mandibul
1	*Corynebacterium sp*		Ba	B	a	Bb	B	B?	0	s	S
2	*Corynebacterium sp*		0	b	0	0	0	0	0	0	0
3	*Corynebacterium sp*		0	0	A	A	A	0	0	0	0
4	*Corynebacterium sp*		0	0	0	ab	0	0	0	0	0
5	*Corynebacterium spp*		0	0	0	0	a?	0	B?	0	0
6	*Corynebacterium sp*		0	0	0	0	0	0	b	0	0
7	*Streptomyces s Nocardia sp*		B	0	0	0	0	0	0	0	0
8	*Streptomyces sp ?*		0	0	0	0	0	0	B	0	0
9	*Fusobacterium sp*		0	0	0	0	0	b	0	0	0
10	*Haemophilus sp ?*		0	b	a	Bb	ABab	0	0	S	s

The types of cultural source are A = aerobic blood agar a = anaerobic blood agar B = secondary streak plate from aerobic blood broth b — secondary streak plate from anaerobic blood broth S = secondary streak plate from aerobic serum ascites broth, s = secondary streak plate from anaerobic serum ascites broth ? signifies that the culture died before isolation and characterization were achieved, 0 signifies that the strain was not isolated

1 *Corynebacterium sp*[a] from biopsies nos 2 through 6, and possibly 7 (the culture died before isolation from no 7) Colonial appearance on blood agar (C) grey white colonies, 1 2 mm in diameter after two days Biochemical reactions (B) facultative anaerobe, catalase positive, fermentation (without gas production) of aesculin, dextrin, D-fructose, and maltose and dubious change of saccharose and salicin, no reduction of nitrate, no liquefaction of gelatin or change of lithmus milk, production of hydrogen sulphide not investigated, indole not produced Microscopical appearance (M) small, straight rods, 0 5 × 1 0–1 5 µm, ends not swollen arranged in bundles or Chinese letters, Gram-positive, uniformly stained, with few metachromatic granules

2 *Corynebacterium sp* from biopsy no 3 C grey, elevated, glistening circular, with an entire edge, less than 1 mm in diameter B facultative anaerobe, catalase positive, gelatin not liquefied, fermentation (without gas production) of aesculin, L-arabinose, arginine, D glucose, galactose, D fructose, inulin, lactose, maltose, raffinose, saccharose, and slightly of glycerol, no reduction of nitrate or production of hydrogen sulphide indole not produced, reduction and coagulation, but no lysis of lithmus milk M rods, 0 5 × 0 8–1 0 µm, uniform in diameter, arranged in bundles or Chinese letters, evenly stained, Gram positive, few but clearly visible, metachromatic granules

3 *Corynebacterium sp* from the 4th, 5th, and 6th biopsies C salmon coloured smooth colonies, 1 mm in diameter after two days B facultative anaerobe, catalase positive gelatin unchanged not fermentative, but hydrolyzes urea, hydrogen sulphide not produced nitrate not reduced lithmus milk unchanged, indole not produced M 0 5 × 1 0 1 6 µm, straight rods without club formation, arranged in bundles or Chinese letters stains uniformly and Gram positively, few metachromatic granules

4 *Corynebacterium sp* from the 5th biopsy C about 1 mm, grey, smooth dome shaped colonies B facultative anaerobe, catalase positive gelatin not liquefied fermentation (gas not produced) of aesculin L arabinose dextrin, D-fructose, lactose, maltose, and saccharose, slight change of inulin, raffinose, and L-rhamnose no reduction of [illegible]
or [illegible]
M [illegible]
bu [illegible] letters uniformly stained Gram positive, few metachromatic granules

5 *Corynebacterium spp* found in biopsies nos 6 and 8 both died before isolation and identification were achieved

6 *Coryneb* [illegible]
grey op [illegible]

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[a] The a [illegible] ederiksen M D Statens Seruminstitut [illegible] firming the diagnosis which could not [illegible]

anaerobe, catalase positive, gelatin liquefied, not fermentative, urea not hydrolyzed, no production of hydrogen sulphide or indole, nitrate not reduced, no change of lithmus milk M pleomorphic, straight or curved rods, about 0 5 × 1 5–3 μm, arranged in bundles or Chinese letters, rather regularly stained, Gram-positive, with few, but prominent metachromatic granules

7 *Streptomyces s Nocardia sp*[1] found in the 2nd biopsy, only C yellow-white, cartilagineous colonies, about 4 5 mm in diameter after four days, with a granular surface and fimbriate edge, no aerial hyphae B aerobe, fermentation (without production of gas) of lactose, L rhamnose, D-xylose, D glucose, dextrin, and, slowly, of maltose, gelatin not liquefied, lithmus milk not changed M branching threads, 0 2–1 0 μm in diameter, Gram-positive, not acid fast

8 *Streptomyces sp* ? from the 8th biopsy, only C irregular, granulated, cartilagineous, yellow, somewhat burrowing colonies, less than 1 mm in diameter in 24 hours B facultative anaerobe, catalase positive, not fermentative, gelatin liquefied, lithmus milk is coagulated and lysed, but not reduced M threads and rods, about 0 5 μm in diameter Gram-positive, not acid fast

9 *Fusobacterium sp* isolated from the 7th biopsy C smooth, domed grey colonies, about 1 mm in diameter, with irregular edge B obligate anaerobe only glucose is fermented M spindle shaped cells 0 5–0 8 × 4–10 μm, occurring singly, in pairs, and in long chains, irregular, Gram negative staining

10 *Haemophilus sp* ?- found in the 3rd through 6th biopsies C colonies just visible (after one to two days) as low convex, circular, colourless, transparent colonies with an entire edge B facultative aerobe, after repeated subcultures facultative anaerobe, dubious catalase reaction growth in enriched media only, but blood not necessary, and no satellism around staphylococcal colonies, no change in colour of blood agar, slow fermentation of D-glucose and aesculin only, nitrate is reduced and indole produced, lithmus milk is reduced, coagulated, and lysed M small rods, 0 2 0 3 × 1 0 μm, not pleomorphic with slight tendency *to polar staining Chemotherapeutic susceptibility* tests, employing the tablet method showed strong inhibition by sulphathiazole, streptomycin, chloramphenicol, erythromycin, polymyxin B, and neomycin + bacitracin less effective were oxitetracycline, penicillin, and Fucidin®

Blood Cultures

In the anaerobic culture at the time of the third biopsy a single colony (from five plates in all) of a *Corynebacterium sp* was found and in the aerobic culture, in connection with the fourth biopsy, growth of a

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1 Cf footnote[1] on p 435
2 Cf footnote[2] on p 437

Micrococcaceae sp occurred Both strains were considered to be contaminants, from the skin as all other aerobic and anaerobic blood cultures, made simultaneously with the second to the fifth biopsy, showed no growth

Teeth Description of Bacterial Strains

The upper teeth were removed on the 89th day and the lower ones on the 95th day By streaking on blood agar plates from the aerobic and anaerobic, primary serum-ascites broth many different strains of microorganisms were isolated and several from each tooth As the technique employed did not permit a strict separation of the *pulpal material* from the *tooth surface* flora, and as the two kinds of cultures yielded much the same strains, only the overall results are given here and in Table 1

In spite of the attempted sterilization by flame of the tooth surface of all the lower teeth, the flora was found to be very similar to that of the upper teeth

The *Corynebacterium sp*, numbered 1 above, was found in cultures from 4+, 8+, —5, and 8—, which teeth all showed parodontitis of apical, marginal or total type

The presumed *Haemophilus sp* was isolated from +5 by aerobic and from 8+ by anaerobic incubation—and from the pulpa and surface, both, these teeth showed apical parodontitis

In addition to the strains indicated in Table 1 the following strains or types, already or later known from the jejunal biopsies, appeared *Micrococcaceae*, *Veillonellae*, *Streptococci* of the viridans, enterococcus and lactis groups *Lactobacilli*, *Clostridia*, *Candida albicans*, and threadlike Gram negative rods (*Leptotrichiae?*)

Dental strains, which were found to be different from strains isolated from the second, third, and fourth biopsies, were not investigated further They comprised escherichia like organisms, *Fusobacteria*, *Staphylococci*, *Neisseriae*, *Diplococcus pneumoniae*, *Corynebacteria*, *Bacilli* threadlike, Gram-positive rods, and large, Gram-negative rods

Histological Examinations

Light Microscopy

The stained, jejunal tissue revealed the well known picture (*Rostgaard* 1964) O[illegible] rods, especial[illegible] le at 1000 × [illegible] The presum[illegible] could be seen in methylene blue and PAS-stained preparations as well as in Gram-stained sections In spite of every effort to vary the differentiating procedure the bacterial masses—already found by electron microscopy—never stained qualitatively different from the surrounding, Gram-negative human cells In some



Fig 2

Electron micrographs from the jejunal mucosa in a case of Whipple's disease 70 000 ×.

Rod-shaped bacteria from the lamina propria of the second jejunal biopsy (before treatment) At left (a) the vacuolated cytoplasm and multi-layered envelope are seen At right (b) the longitudinal section of a presumably dividing bacterium and several cross sections are found

sections extraneous bacteria and the contents of the epithelial goblet cells stained Gram-positively, the cells otherwise Gram-negatively, and yet the clumps of bacteria in the lamina propria showed Gram-negativity

Electron Microscopy[3]

At 70 000 × magnification (cf Fig 2) the best preserved bacterial bodies from the second biopsy, before the administration of antibiotics, revealed a maximal size of 0 20 × 1 1 μm for cells without evidence of incipient division The interior was of varying electron density with irregular "vacuoles" The envelope consisted of several layers, having a maximal total thickness of about 32 0 nm[4] By close inspection at least three light layers separating four darker membranes could be discerned This might be described as an inner double layer, the cytoplasmic membrane (about 6 5 nm thick), with one membrane lying close upon the cytoplasm and the other adhering to the inside of the electron dense cell wall (about 7 5 nm thick), which in itself is double layered and separated by a thin space from the capsular layer (about 17 0 nm in thickness) In presumably dividing bacteria no septa could be seen

DISCUSSION

Whipple (1907) described small rods, about 0 2 × 1–2 μm, found by Levaditi impregnation of a lymph node, and he discussed their possible bacterial nature This observation was not duplicated until recently (*Yardley & Fleming II* 1961) At present, however, the electron microscope permits an identification of the rods as bacteria (*Chears & Ashworth* 1961, *Yardley & Hendrix* 1961, *Kurtz, Davis, Jr & Ruffin* 1962, *Caroli, Strolin & Julien* 1963d, *Rostgaard* 1964)

Irrespective of the possible aetiological rôle of the bacteria in the jejunal mucosa and lymph nodes, i e, whether they are a fortuitous occurrence or act in a primary or secondary way, it would be of interest to isolate the microorganisms if they be viable

Earlier attempts at incriminating bacteria as important agents in Whipple's disease are summarized in Table 2 Apart from probably non-specific results by *Whipple* (1907) and *Gross* (1959), no small, rod-shaped bacteria have been isolated before the recent reports by *Caroli et al* (1963b, c) and the present one

In the present case many kinds of bacteria and a *Candida albicans* could be isolated from seven of the eight peroral, jejunal biopsies (no cultures were made from the first) As the open capsule must pass through the mouth pharynx, and a length of small intestine prior to activation of the mechanism, it is not surprising that the biopsies yield numerous and varying strains

The first attempt at cultivation, i e from the second biopsy, was not up to the later standard, and, consequently, the results are not fully representative From the third biopsy onwards the search became more

[3] The authors gratefully acknowledge the help rendered by Mr *A Birch Andersen* M Sc Statens Seruminstitut (Department of Biophysics), in interpreting the electron micrographs

[4] 1 nm (one nanometer) = 10^{-9} m = 10 Angstrom



Fig 2

Electron micrographs from the jejunal mucosa in a case of Whipple's disease
70 000 ×

Rod shaped bacteria from the lamina propria of the second jejunal biopsy (before treatment) At left (a) the vacuolated cytoplasm and multi layered envelope are seen At right (b) the longitudinal section of a presumably dividing bacterium and several cross sections are found

sections extraneous bacteria and the contents of the epithelial goblet cells stained Gram positively, the cells otherwise Gram negatively and yet the clumps of bacteria in the lamina propria showed Gram negativity

thorough and special emphasis was laid on the examination of the rodlike bacteria The strains which might conceivably look like the rods found in the electron micrographs are summarized in Table 1, and the chronological relationship between biopsies and therapy is shown in Fig 1

Table 1 reveals that *Corynebacteria* nos 4, 5, and 6, the *Streptomyces sp?* (8), and *Fusobacterium sp* (9) all were isolated from late and single biopsies Consequently, these strains are probably not of the type encountered in the jejunal mucosa The seventh species (*Streptomyces s Nocardia sp*) is an unlikely suspect, too, as its morphology differs very much from the uniform picture in the electron micrographs The culture shows branching threads of varying diameter, from 0 2–1 0 μm The chance of missing the third strain of *Corynebacteria* in two biopsies and then isolate it from three consecutive biopsies is rather small, if it were the culprit, and furthermore this strain has cells which are rather thick about 0 5 μm The second strain of *Corynebacteria* might have been missed in the second biopsy and found in the third, but if the strain survived the first period of antibiotic treatment it would be expected in the fourth biopsy, which was taken before the second period of treatment Thus, the most suspicious strains seem to be the first *Corynebacterium sp* and the *Haemophilus sp ?* Before a choice is made the results by *Caroli* and coworkers shall be discussed in detail

Caroli Prevot, Julien, Sébald & Éleve (1963c) and *Caroli, Prevot, Julien, Gueritat & Strolin* (1963b) cultivated *Corynebacterium anaerobium* and lesser numbers of two other unspecified kinds of bacteria in cultures from an inguinal lymph node The node was extirpated two days after the onset of the antibiotic therapy in a patient with a typical *Whipple s* disease By microscopy the lymph node was found to contain large numbers of Gram positive bacteria After seven days of treatment a laparotomy permitted culturing from a jejunal biopsy, mesenteric lymph nodes and ascitic fluid All cultures proved sterile, and the authors attribute these last results to the effect of the treatment

The *strain of C anaerobium* was anaerobic or microaerophilic and did not require serum This bacterial species is known to show a predilection for the reticulo-endothelial system (*Prevot* 1960)

The *electron* micrographs made by *Caroli et al* (1963d) show well preserved bacteria, 0 25 × <1 8 μm, which are described as having a cytoplasm surrounded by a double layered, cytoplasmic membrane, 5 6 nm[1] thick a dense homogenous cell wall, 9–10 5 nm in thickness, and a capsular layer, 16–19 nm thick giving a total thickness of the membrane system of 30–35 *nm*/These measurements may be controlled from the beautiful reproductions in the report Referring to a paper by *Glauert* (1962) the authors *consider* these measurements to be typical of Gram positive organisms Furthermore, the Gram stained, jejunal

[1] Cf footnote[4] on p 431

TABLE 2

Some Results of Cultural and Serological Investigations in Whipple's Disease as Reported in the Literature

Reference	Cultural examination of							Serological investigations for antibodies against			
	Jejunal biopsy	Mesenteric lymph nodes	Peripheral lymph nodes	Parenchymatous organs	Blood	Peritoneal exsudate	Pathogenic bacteria in stools	*Salmonellae* (Widal)	*Brucellae* (agglutination)	*Streptococci* (antistreptolysin titre)	*Leptospirae* (Weil)
Whipple (1906)	–	+ a)	0 b)	+ c)	–	–	–	–	–	–	–
Clemmesen (1945)		–		–	0	–	0	0	–	450	–
Hendrix & al (1950)		0		–	0	–	0 d)	0	0	–	–
Peterson (1951)		–		–	0 e)	–	–	–	–	–	–
Russo (1952)	–	–		–	–	–	–	0 d)	0 d)	–	–
Upton (1952)	–			0	–	–	–	–	–	–	–
Jorgensen (1954)				–	–	–		–	–	320	–
Radding & al (1954)				–	–		0	–	–	–	–
Bie (1955)				–	–	–	–	0	–	–	0
Puite & al (1955)			0	–	0	–	0	0 f)	0 f)	–	0
Schaffner & al (1955)	–	–			–	–	–	0	–	4000	–
Ammann (1957)	–				–	–	–	0	0	–	–
Gross & al (1959)	–	0	0 d)	–	0	0	+ g)	–	–	–	–
England & al (1960)		–		–	–	–	0	–	–	–	–
Caroli & al (1963b)	0	0	+ h)	–	–	0	0 d)	–	–	–	–
Present case (1964)	+		–	–	0		0	50 i)	0	125 j)	0

a) coli like organisms
b) subcutaneous inoculation of a lymph node in a rabbit resulted in a small subcutaneous abscess and the animal died after seven weeks from pneumonia. No mycobacteria could be demonstrated in a lymph node by subcutaneous inoculation on a rabbit
c) white coccus of low virulence
d) in two cases
e) in three cases, two of which were also investigated by inoculation without reaction
f) in three cases
g) *Aerobacter aerogenes, Staphylococcus aureus, Proteus sp.*
h) *Corynebacterium anaerobium* and two other kinds of (unspecified) bacteria
i) this anti H titre was explained by earlier vaccination
j) additionally the anti streptococ hyaluronidase titre was 5000 (doubtfully positive)
+ signifies isolation of microorganisms, 0 signifies no growth, – signifies no data

TABLE 3

The Biochemical Properties of Some Strains of Corynebacteria

A = *C. anaerobium* isolated by *Caselitz et al.* (1963c) from an inguinal lymph node in a case of Whipple's disease. B = The original description of *C. anaerobium* by *Prévot* (1960). 1–5 = The pure strains of *Corynebacteria* from the several jejunal biopsies in the present case (cf. Table 1)

Strain	Oxygen requirement	Catalase reaction	Colour of colonies	Liquefaction of gelatin	Fermentative powers acid gas	Property: Fermentation of Glucose	Fructose	Maltose	Galactose	Glycerol	Milk coag. lysis	Production of indole	Production of hydrogen sulphide	Reduction of nitrate
A	ana m.a.	+	salmon	0	+/0	+	+	+	+	+	+/			+
B	ana or m.a.	0 or aq.	salmon	0	+/0						+/	0 or +	0	+
1	f.a.	+	grey white	0	+/0	0	+	+	0	0	0/	0		0
2	f.a.	+	grey	0	+/0	+	+	+	+	(+)	+/0	0	0	0
3	f.a.	+	salmon	0	0/	0	0	0	0	0	0/	0	0	0
4	f.a.	+	grey	0	+/0	0	+	+	0	0	0/	0	0	0
5	f.a.	+	grey	+	0/	0	0	0	0	0	0/	0	0	0

+ = positive reaction; (+) = slight change; 0 = no effect; — = not investigated or not recorded; sign / signifies that a previous [illegible] no effect excludes the possibility; f.a. = facultative anaerobe; ana = obligate anaerobe; m.a. = microaerophile

mucosa revealed Gram-positive granules extra- and intracellularly, which finding confirms earlier reports by *Yardley & Hendrix* (1961), *Chears, Jr & Ashworth* (1961), and *Kurtz, Davis, Jr & Ruffin* (1962)

Glauert's review (1962) of the fine structure of bacteria minutely describes the cell wall proper—*i e* excluding the cytoplasmic membrane and surface layers (slime or capsule) In Gram-negative bacteria the thickness of the cell wall is about 7 5 nm: it is double-layered, consisting of two electron-dense layers separated by a less dense layer The Gram-positive bacteria, in contrast, possess a wall, 15–35 nm or more in thickness, having two thin, dense layers separated by a wide layer of less dense material

Judging from these results, the bacteria in *Caroli* and coworkers' electron micrographs, with a cell wall proper of 9–10 5 nm in thickness, might be Gram-negative rather than Gram-positive, furthermore, the description of the cell wall as homogenous perhaps points to Gram-negativity. as the thin, less electron-dense, intermediate layer, characteristic of Gram-negative bacteria, might be invisible for technical reasons

In electron micrographs from the present case (Fig 2) the maximal size of the bacteria is 0 2 × 1 1 µm, and they may be described as having a double-layered cytoplasmic membrane of 6 5 nm, a double-layered cell wall of 7 5 nm, and a capsular layer of 17 nm These measurements tally very closely with those cited above from the report by *Caroli et al* As a further sign of possible Gram-negativity no preceding cross walls could be discerned in presumably dividing cells

Due to the several reports of Gram-positive granules in the jejunal lamina propria in Whipple's disease repeated attempts were made at demonstrating this character in paraffin embedded sections, the bacterial clumps, however, always stained with the same quality of colour as the surrounding tissue—even in sections where the goblet cell mucus and some extraneous cocci showed Gram-positivity

Returning to the earlier, most suspicious, bacterial strains, one is a Gram-positive *Corynebacterium sp* (no 1), which in culture is 0 5 × 1 0–1 5 µm, the other is Gram-negative, resembles *Haemophili*, and measures 0 2–0 3 × 1 0 µm The first is too thick to resemble the electron micrographical descriptions the last appears a better morphological possibility, and the Gram-negativity parallels the findings in the present case As will be seen from Table 3, none of the *Corynebacteria* isolated from the jejunal biopsies fit either the original description of *C anaerobium* (*Prevot* 1961) or the strain isolated by *Caroli et al* (1963b)

If the bacteria in the jejunal mucosa from the two patients are of the same type—as suggested by the electron micrographs—the alternatives for the present are the *Corynebacterium anaerobium*, found by *Caroli* and coworkers, and the *Haemophili*-like organism from the present case The final decision is difficult for several reasons

preference The presence of a thin capsular layer does not speak against *Haemophili* which often possess capsules in the pathogenic state

Detection of Bacteria in Relation to Therapy

It might be argued in the present case that the only assay at cultivation before antibiotic therapy—from the second biopsy—was inadequate and consequently a *Corynebacterium sp* was missed and later killed by treatment The strain resembling *Haemophili* then would be called an irrelevant contaminant and this argument might be substantiated by the repeated isolation of the strain from the second to the sixth biopsy inclusive—this in spite of antibiotic treatment for protracted periods The latter argument does not hold because penicillin and tetracycline which were mostly used before the time of the sixth biopsy were shown to be a suboptimal combination against the Gram negative small rods Following the subsequent start of continuous sulphonamide medication no such bacteria were isolated from two biopsies *Caroli Julien Éteve Prevot & Sebald* (with *Stralin Gueritat & Cadore*) (1963a) illustrate that the choice of chemotherapeutics is extremely important and that the optimally effective drugs vary from case to case and perhaps from time to time in a given case With suboptimal therapy and in spite of clinical improvement it might be possible still to isolate the pertinent organism surviving as resting forms for long periods This possibility is supported by the light microscopical presence of decreasing numbers of minute rodlike PAS positive granules in or adjacent to the epithelial basal membrane of the six first jejunal tissue specimens no rods appeared in the eighth biopsy (the seventh was not examined histologically)

CONCLUSIONS

The well known Henle Koch criteria for the establishment of a microorganism as aetiology of a disease are repeated isolation of the microbe fro n
cultivation of the organism *in vitro*
n healthy subjects resulting in pro

t fulfillment of the last requirement may prove impossible in Whipple's disease if animals are not susceptible furthermore the prodromal articular and polyserosal symptoms point to a long period of development before the typical syndrome is established A special susceptibility even may be required

The final proof of the aetiology is probabl
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Material for Cultivation

The biopsies from the jejunum suffer from the unavoidable contamination *en route*. Thus, the *Haemophili* might be part of the oral microflora, swabs from a deep caries at the time of biopsy no. 3 (54th day), and from the same caries and the necks of several premolars at the time of the fourth biopsy (66th day) were incubated, but did not reveal this strain—this negative result, of course, does not exclude the possibility of *Haemophili* in the mouth. In the case of *Caroli et al.* (1963b) the inguinal lymph node from a very ill patient could be irrelevantly contaminated, too, as it receives the lymph from the leg, the authors even mention the isolation of two other microorganisms, besides the *Corynebacteria*.

Isolation

The primary isolation of the nutritionally very demanding *Haemophilus sp.?* always proved rather difficult, and the extremely small, colourless, transparent colonies are readily missed. During the first cultural efforts (biopsy no. 2) in the present case this strain might easily have been overlooked. In the same biopsy, on the other hand, a *C. anaerobium* might have remained undetected, and subsequently been killed by chemotherapy.

Gram-Staining

If the organisms from the two cases are of the same type, it is difficult to explain the above discrepancy between the Gram-positive bodies in the jejunal tissue, as found by several authors, and the consistent Gram-negativity in the present case—in spite of abundant PAS-positive material in the lamina propria. Incidentally, the failure of demonstrating Gram-positivity does not argue definitely against *Corynebacteria* as these are Gram-labile, and, furthermore, the Gram technique is often difficult to perform on paraffin embedded tissue sections. Also, the defences of the *macroorganism might have weakened any inherent* Gram-positivity of the bacteria.

Electron Micrographs

Electron micrography is influenced by technical as well as biological factors, and the interpretation is always difficult. The final result depends upon the fixation and embedding, and the surrounding tissue may impede the optimal presentation of bacteria. However, the pictures by *Caroli et al.* (1963b) are very clear, and their results tally very closely with the measurements in the present case. With reference to the work by *Glauert* (1962) it has been discussed above, why the micrographs can be interpreted as showing Gram-negative bacteria, but the designation of the different layers is, of course, often a matter of

preference The presence of a thin, capsular layer does not speak against *Haemophili*, which often possess capsules in the pathogenic state

Detection of Bacteria in Relation to Therapy

It might be argued, in the present case, that the only assay at cultivation before antibiotic therapy—from the second biopsy—was inadequate, and consequently, a *Corynebacterium sp* was missed and later killed by treatment The strain resembling *Haemophili*, then, would be called an irrelevant contaminant, and this argument might be substantiated by the repeated isolation of the strain from the second to the sixth biopsy, inclusive—this in spite of antibiotic treatment for protracted periods The latter argument does not hold, because penicillin and tetracycline, which were mostly used before the time of the sixth biopsy, were shown to be a suboptimal combination against the Gram-negative, small rods Following the subsequent start of continuous sulphonamide medication no such bacteria were isolated from two biopsies *Caroli, Julien, Éteve, Prevot & Sebald* (with *Stralin, Gueritat & Cadore*) (1963a) illustrate that the choice of chemotherapeutics is extremely important, and that the optimally effective drugs vary from case to case, and, perhaps, from time to time in a given case With suboptimal therapy, and in spite of clinical improvement, it might be possible, still, to isolate the pertinent organism surviving as resting forms for long periods This possibility is supported by the light microscopical presence of decreasing numbers of minute, rodlike, PAS-positive granules in or adjacent to the epithelial basal membrane of the six first jejunal tissue specimens, no rods appeared in the eighth biopsy (the seventh was not examined histologically)

CONCLUSIONS

The well known Henle-Koch criteria for the establishment of a micro organism as aetiology of a disease are repeated isolation of the microbe from several cases of the disease, cultivation of the organism *in vitro*, inoculation of cultural material on healthy subjects, resulting in production of the original syndrome

The fulfillment of the last requirement may prove impossible in Whipple's disease, if animals are not susceptible furthermore, the prodromal articular and polyserosal symptoms point to a long period of development before the typical syndrome is established A special susceptibility, even, may be required

The final proof of the aetiology is probably elusive, and we may have to rely entirely on circumstantial evidence The presence of bacteria in the jejunal tissue is beyond question, and their importance is proved by the effect of antibiotics The strain of *Corynebacterium anaerobium* isolated by Caroli and coworkers (1963b, c), possibly, is the cause of

the disease— at least in one case However, the electron micrographs seem to permit a minute, Gram-negative rod as aetiological agent One strain of *Haemophili*-like bacteria, isolated from repeated, jejunal biopsies in the present case, appears to fit the findings better than *Corynebacteria* Serological studies have been undertaken in order to collect further information

SUMMARY

The presence of bacteria in the jejunal mucosa of a patient with verified Whipple's disease is demonstrated by electron microscopy (cf also the following report) Repeated, peroral, jejunal biopsies and material from extracted teeth yield a multitude of different microbial strains by aerobic and anaerobic incubation on enriched media Most strains seem to be contaminants, but two remain as possibly identical with the slender, short rods in the jejunal mucosa a *Corynebacterium sp* and a *Haemophili*-like organism The reports by *Caroli* and co workers are reviewed, they confirmed earlier findings of Gram positive granules in the jejunal tissue, and these authors cultivated a *Corynebacterium anaerobium* from an inguinal lymph node of a patient with Whipple's disease, furthermore, they described presumably Gram-positive bacteria by electron micrography In the present case Gram-negative rods, only, could be seen by light microscopy of stained tissue, and the electron micrographs may be interpreted as showing Gram-negative bacteria—in shape and size resembling the *Haemophili sp ?* from the cultures None of the several *Corynebacteria* isolated from the present case correspond morphologically or biochemically to *C anaerobium* The *Haemophili*-like strain is primarily a facultative aerobe, changing to a facultative anaerobe by repeated subcultivation, it is nutrionally very requiring, and grows in barely visible colonies It was but moderately affected by penicillin and oxitetracycline *in vitro,* which might explain why it was possible to isolate the strain from repeated, jejunal biopsies—in spite of the clinical improvement of the patient During therapy with more active drugs the strain was not found in two biopsies

The difficulties of proving which microorganism (if any) is the aetiological agent in Whipple's disease are discussed, and the *Haemophili* like rod might be a better possibility than *Corynebacteria*

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FROM DEPARTMENT OF ANATOMY (CHIEF PROFESSOR DR MED HARALD MOE) THE ROYAL DENTAL COLLEGE UNIVERSITETSPARKEN 4 COPENHAGEN Ø DENMARK

BACTERIA IN WHIPPLE'S DISEASE

2 *Combined Electron and Light Microscopy*

By

J ROSTGAARD

Received 19 viii 63

Attempts to establish the aetiology and pathogenesis of Whipple's disease have been almost as numerous and varied as the reports of cases (see review by *Rutishauser & Borer* 1959) The use of peroral intestinal biopsy coupled with new techniques of enzyme histochemistry and electron microscopy have shed some light on the nature of the lesion in the small intestine In 1949 *Black–Schaffer* called the attention to an intracellular PAS-positive substance which he presumed to be a glycoprotein It is believed that this PAS-positive material is of major significance in the pathogenesis of the disease By electron microscopy, *Chears & Ashworth* 1961, *Yardley & Hendrix* 1961, *Cohen* 1962, *Kurtz, Davis & Ruffin* 1962, and *Caroli et al* 1963, recently described the fine structure of some intracytoplasmic particles and provided substantial evidence of their bacterial nature These authors suggested a relationship between these bacteria and the PAS-positive granules seen in macrophages in this disease Three other case reports (*Haubrich, Watson & Sieracki* 1960, *Cohen, Schimmel, Holt & Isselbacher* 1960, and *Hollenberg* 1962) utilizing electron microscopic inspection of the jejunal mucosa failed to identify these same particles as bacterial In the present paper ultrastructural findings in tissues obtained by serial biopsies from a patient with Whipple's disease will be described and compared with those obtained by light microscopy The findings support the theory that Whipple's disease may be either a peculiar infection due to

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The author wishes to thank Professor *K Brochner Mortensen* Medical Department A of the University Hospital Copenhagen for permission to investigate this case A part of … was performed in the Laboratory for Electron Micr… of Copenhagen The author is greatly indebted t… *Dahl* for their hospitality My thanks are due to Mr… [which] valuable advice to Professor *Harald Moe* for making several helpful suggestions The technical assistance of Miss *K Sjoberg* is gratefully acknowledged

a microorganism most closely resembling a bacterium, or, even if the organisms are not the primary causative factor, that they may play a rôle in the genesis of the PAS-positive granules

MATERIALS AND METHODS

Source of tissue Peroral jejunal biopsies, obtained by the instrument devised by *Crosby & Kugler* (1957), were taken from a 34 year old male with histologically [illegible]

[illegible]

of the biopsy specimens The results of these bacteriological studies and the case report are published elsewhere (*Kok, Dybkær & Rostgaard* 1964)

Portions of fresh jejunal tissues were fixed immediately in buffered osmium tetroxide according to the formula given by *Sjöstrand* (1956) and embedded in Vestopal as described by *Ryter & Kellenberger* (1958) Alternating semi thick (1 micron) and ultrathin sections were cut with a glass knife on the LKB microtome The thin sections were stained with uranyl acetate and studied in a Philips electron microscope (EM 100B) or in a Siemens Elmiskop I Staining including the PAS-reaction, of the semi thick sections for light microscopy were carried out with the "grid drop" techn[illegible]

pu [illegible]
me [illegible]
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af [illegible]

[illegible] washed, and mounted in balsam

RESULTS

Light Microscopy

Examinations of sections of the small intestinal mucosa stained with *hæmatoxylin and eosin* show changes typical for Whipple's disease The villi are distended and distorted, but the epithelial cells are of normal appearance The lamina propria is packed with many mononuclear cells measuring as much as 40 micra in diameter The abundant cytoplasm *of the cells* is of a pale, eosinophilic, foamy appearance The lamina muscularis mucosae is normal PAS-staining shows an intense positive *reaction in the* lamina propria The major portion of the PAS-positive material is found inside the mononuclear cells and located in 1 to 3 microns *granules* their staining intensity varies over a wide range (Fig 1) These cells remain PAS-positive after treatment with diastase *and pectinase* (*McManus & Mowry* 1960) It is of interest to note that in the biopsy obtained 16 months after initiation of antibiotic therapy, *a decrease in the* number of PAS-positive cells in the lamina propria is seen for the first time, This is in accordance with *Davis*,

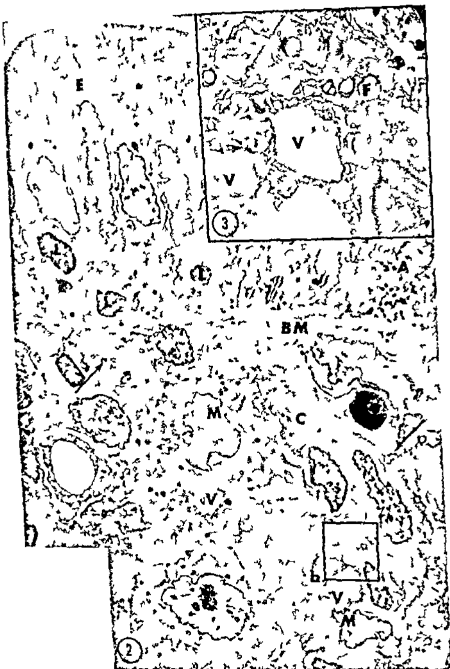
Fig 1

Light photomicrograph of a peroral jejunal biopsy from a patient with Whipple's disease showing epithelial cells (F) and lamina propria from a villus PAS stained, 1 micron section of the type used for correlative light and electron microscopy
hown in Figs 2
deeply stained
epithelial base
en the striated
border and the epithelial nuclei are numerous PAS positive cytoplasmic bodies × 1 800

Figs 2 3

Fig 2 Electron micrograph of area a Fig 1 The large PAS-positive granules found inside the macrophages (M) are readily apparent as vacuoles (V) in the cytoplasm Tightly packed clumps of "minute bodies" (arrows) are present in the lamina propria beneath the basement membrane (BM) of the mucosal epithelium (F) and along an adjacent capillary (C) One argentaffine cell (A) and several lymphocytes (L) are seen in the epithelium at the top of the micrograph × 2,850

Fig 3 Electron micrograph of area b Fig 1 and area b Fig 2 showing portion of



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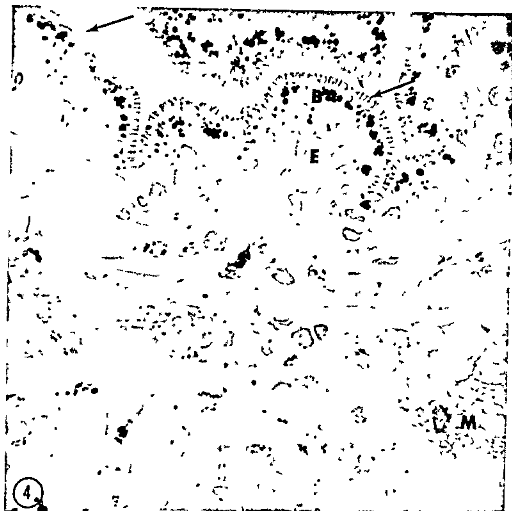
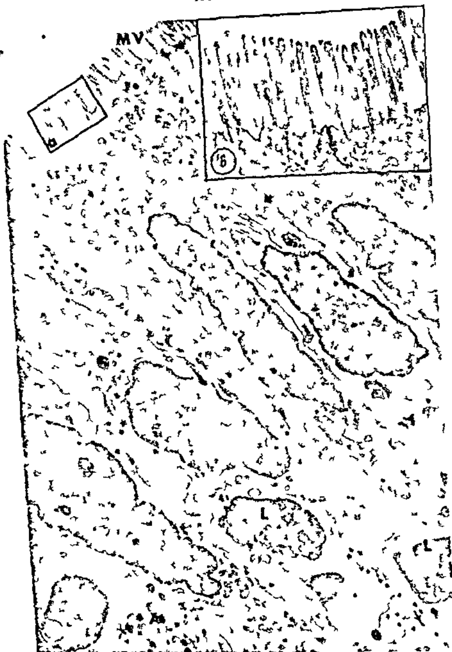


Fig 4

Light photomicrograph of 1 micron thick section of a jejunal biopsy specimen obtained about one year after initiation of clinically successful therapy showing acid phosphatase activity in epithelial cells (E) and in lamina propria from a villus The reaction product is located in cytoplasmic bodies (B) in the luminal portions of the epithelium and in some parts of the striated border but not where the surface of the cells are relatively free (arrows) No acid phosphatase activity is found in the foamy mononuclear cells (M) typical for Whipple's disease but is present in several other cells in lamina propria × 900

McBee, Borland, Kurtz & Ruffin (1963), since they reported that a decrease in number of PAS-positive cells first appeared 2 years after initiation of the antibiotic treatment In addition to the intracellular PAS-positive material a very fine, granular, PAS positive material is revealed outside the macrophages, along the basement membrane of the epithelial cells and around the capillaries These extracellular granules are small, just within the resolution of the light microscope, and are not observed in biopsy specimens obtained after antibiotic therapy has been initiated By correlative light microscopy of thick sections and electron microscopy of the same field in adjacent thin



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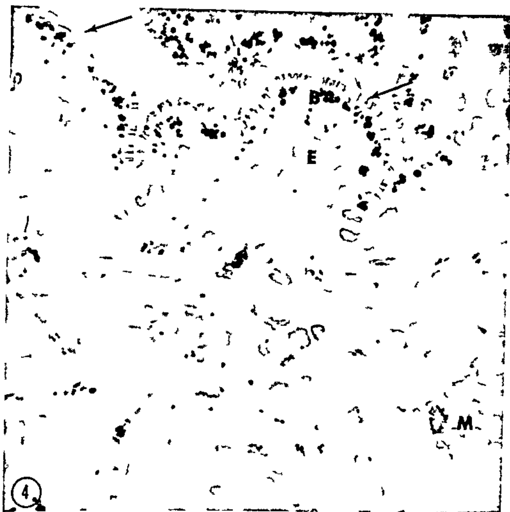


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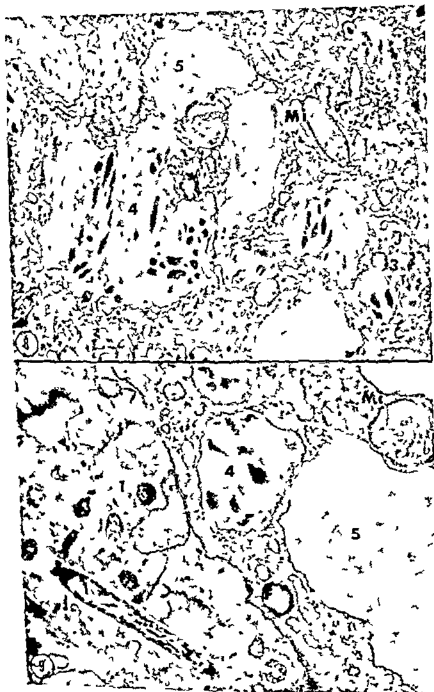

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Fig 7

Electron micrograph of a m n ite l od in greater magnif ati n ho s ng the fine l tails cl aracteri tic of l acteria (B) The l acteri im is lying free in the inter t t al sl ace in lamina j roj r a ji st l eneath tl e l asement meml rane (BM) A j ortion of an argentaffine cell (A) an l an absorj t ve cell mitoch n lri n (Mi) are seen × 80 000

sections it is possible to recognize the extracellular granules as tightly packed clumps of bacteria (Fig 2) The alcian blue method for acid mucopolysaccharides demonstrates the intracellular granular material to be intensely positive and the extracellular fine granular material to be faintly positive The extracellular fine granular material is Gram negative This is not in agreement with earlier reports (*Chears & Ashworth* 1961 *Yardley & Hendrix* 1961 *Kurt Davis & Ruffin* 1962 and *Caroli et al* 1963) Besides the material is not acid fast In the

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F s 8 an l 9

ea of th lamina j r j r l k lat l r j rti n f n r j l k nt n ng s tl a mixti r f r lat l nt t l a t r a tru ti res at vari i t k f l gra lat n j ar at the r last tak t l lm te l (4) II t t ta l a tl in iter meml rane and t conta a lel cate n t rk f m n l ran Th c rcular forms are cro s sections of ba t r a M toel n lria M l at lr j let (F Fig 8 × 19 000 an l F g 9 × 58 000

propria acid phosphatase activity is found in several cells, but no activity is detectable in the foamy mononuclear cells typical of Whipple's disease This rather distinct localization of the acid phosphatase activity is not in accordance with a report by *Hollenberg* 1962 He found the acid phosphatase to be diffusely distributed both in the mucosal epithelium and the lamina propria

Electron Microscopy

The epithelial cells are of normal appearance in all of the tissue examined with the electron microscope as is also the epithelial basement membrane (Figs 5 and 6) Lymphocytes are frequently found between the epithelial cells Sections of tissue obtained prior to the initiation of the antibiotic therapy show the presence of large numbers of "minute bodies" in the lamina propria (Fig 2) These "minute bodies" are noted at all levels of the lamina propria but are generally concentrated in tightly packed groups just beneath the epithelial basement membrane and around the capillaries (Fig 2) With greater magnification the "minute bodies" clearly show all the fine details characteristic of bacteria (Birch-Andersen 1963) (Fig 7) The greatest length of bacteria measured in the electron micrographs is slightly more than 1 micron Their width is uniform, measuring 0 15 to 0 20 micron Various stages of division are seen (Fig 9) The macrophages contain similar, bacteria-like structures at all stages of degradation These vary in appearance from single, intact bacteria, or vacuoles which contain a mixture of relatively intact forms, to vacuoles containing nothing but possibly the walls of these microorganisms (Figs 8 and 9) By correlative light microscopy of semi-thick sections and electron microscopy of the same field in adjacent thin sections it is possible to show that these vacuoles are the PAS positive granules found inside the macrophages (Figs 1, 2 and 3) At the last stages these intracytoplasmic vacuoles appear to be limited by a thin outer membrane and to contain a delicate network of membranes which probably are the remnants of the bacterial walls (Figs 3, 8 and 9) A few osmiophilic fat droplets may be noted within some macrophages and greater accumulation of fat droplets may be found in others (Figs 3 and 10) Only the outermost part of these droplets are fixed by osmium tetroxide, permitting the more centrally located parts to be dissolved during the dehydration process It is also possible in the fat containing cells to find bacteria at different stages of degradation and vacuoles which contain a mixture of relatively intact forms to vacuoles containing nothing but possibly the walls of the bacteria (Fig 10) Bacteria were not seen in the epithelial layer Electron micrographs of sections from the biopsies, taken following initiation of antibiotic therapy reveal no intact microorganisms However, the macrophages show cytoplasmic vacuoles containing a network of membranes and fat droplets

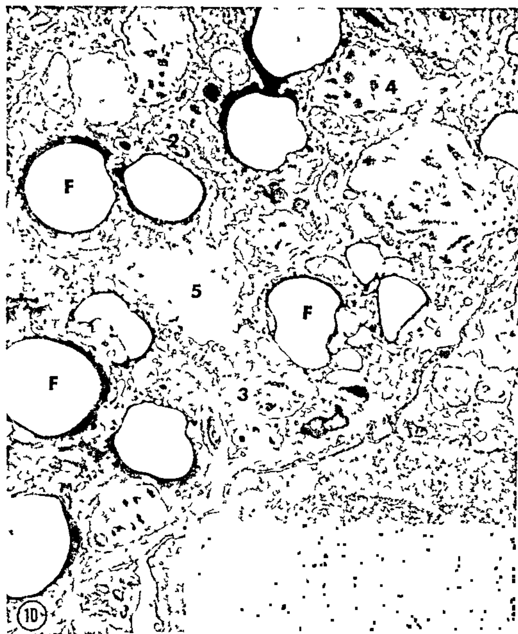


Fig. 10

Electron micrograph of a macrophage from a case of Whipple's disease. Part of the cytoplasm contains numerous bacteria at different stages of degradation (2, 3, 4, 5) and a great accumulation of fat droplets (F). × 20 000

biopsy specimen obtained after one year of antibiotic therapy acid phosphatase activity is localized in cytoplasmic bodies in the luminal portions of the epithelium. In addition a slight reaction is seen in the striated border, but only in the areas where the luminal surfaces of the cells are apposed to one another as *e.g.* between neighbouring villi and in crypts. In areas where the luminal surface of the cells are relatively exposed no positive reaction is found (Fig. 4). In the lamina

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DISCUSSION

This combined light and electron microscopy study adds support to recent reports (*Chears & Ashworth* 1961, *Yardley & Hendrix* 1961, *Kurtz, Davis & Ruffin* 1962, *Cohen* 1962 and *Caroli et al* 1963) that the PAS-positive material found in the macrophages originates from a bacterial organism associated with Whipple's disease and is neither fat nor abnormal glycoprotein present as a manifestation of an altered metabolism, as postulated by *Black-Schaffer* (1949) and *Hollenberg* (1962) The fact that no acid phosphatase activity could be revealed in the "foamy" mononuclear cells indicates that these cells are not actively phagocytizing at this time (about one year after clinical remission had been induced by antibiotics) Unfortunately this reaction has not been carried out on biopsy specimens obtained before the antibiotic therapy had been initiated It is not possible on the basis of the findings in this work to clearly define the rôle of the microorganisms in the aetiology and course of Whipple's disease Nevertheless, it may be useful to point out the agreement between the recent reports mentioned above and the findings reported in this study, particularly the size and ultrastructure of the microorganisms

SUMMARY

Serial, peroral jejunal biopsies were obtained from a case of Whipple's disease before and during one year of a clinically successful, antibiotic and steroid therapy, they were studied with light and electron microscopy For light microscopy, techniques included haematoxylin and eosin, PAS, Giemsa, alcian blue, Gram's stain and Gomori's method for acid phosphatase Characteristic macrophages containing PAS positive granules were found in the lamina propria In a biopsy obtained 16 months after initiation of the antibiotic therapy a decrease in the number of PAS positive cells in the lamina propria is seen for the first time In addition to the intracellular PAS positive material a very finely granular PAS positive material was found outside the macrophages Using light microscopy of semi thick sections and electron microscopy of the same field in adjacent thin sections it was possible to recognize the extracellular granules as tightly packed clumps of bacteria In addition it was possible to demonstrate the fine structure of the PAS-positive granules inside the macrophages The macrophages were seen to contain bacteria like structures at all stages of degradation These included single, intact bacteria, vacuoles which contained a mixture of relatively intact bacteria, and vacuoles containing nothing but possibly the walls of these microrganisms This study adds support to the suggestion that there may be a relationship between bacteria and the PAS-positive macrophages in Whipple's disease

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DISCUSSION

This combined light and electron microscopy study adds support to recent reports (*Chears & Ashworth* 1961, *Yardley & Hendrix* 1961, *Kurtz, Davis & Ruffin* 1962, *Cohen* 1962 and *Caroli et al* 1963) that the PAS-positive material found in the macrophages originates from a bacterial organism associated with Whipple's disease and is neither fat nor abnormal glycoprotein present as a manifestation of an altered metabolism, as postulated by *Black-Schaffer* (1949) and *Hollenberg* (1962) The fact that no acid phosphatase activity could be revealed in the "foamy" mononuclear cells indicates that these cells are not actively phagocytizing at this time (about one year after clinical remission had been induced by antibiotics) Unfortunately this reaction has not been carried out on biopsy specimens obtained before the antibiotic therapy had been initiated It is not possible on the basis of the findings in this work to clearly define the rôle of the microorganisms in the aetiology and course of Whipple's disease Nevertheless, it may be useful to point out the agreement between the recent reports mentioned above and the findings reported in this study, particularly the size and ultrastructure of the microorganisms

SUMMARY

Serial, peroral jejunal biopsies were obtained from a case of Whipple's disease before and during one year of a clinically successful, antibiotic and steroid therapy, they were studied with light and electron microscopy For light microscopy, techniques included haematoxylin and eosin, PAS, Giemsa, alcian blue, Gram's stain and Gomori's method for acid phosphatase Characteristic macrophages containing PAS-positive granules were found in the lamina propria In a biopsy obtained 16 months after initiation of the antibiotic therapy a decrease in the number of PAS-positive cells in the lamina propria is seen for the first time In addition to the intracellular PAS-positive material a very finely granular PAS-positive material was found outside the macrophages Using light microscopy of semi-thick sections and electron microscopy of the same field in adjacent thin sections it was possible to recognize the extracellular granules as tightly packed clumps of bacteria In addition it was possible to demonstrate the fine structure of the PAS-positive granules inside the macrophages The macrophages were seen to contain bacteria-like structures at all stages of degradation These included single, intact bacteria, vacuoles which contained a mixture of relatively intact bacteria, and vacuoles containing nothing but possibly the walls of these microrganisms This study adds support to the suggestion that there may be a relationship between bacteria and the PAS-positive macrophages in Whipple's disease

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BRIEF REPORT

BONE MARROW LYMPHOCYTES IN SPLENECTOMIZED GUINEA PIGS

By *I. Grunnet* and *F. Bierring*

Previous experiments (*Bierring & Grunnet* 1964) have shown that splenectomy in rats causes an increase in the bone marrow content of lymphocytes. The mechanism responsible for the increase has not been established. Thus it is unknown whether it is due to a loss of a specific influence of the spleen on the bone marrow, to a compensatory hyperplasia of lymphoid tissue elsewhere or to secondary factors. The strain of rat used in the experiments carried a latent infection with *Bartonella muris ratti*. The spleen specifically protects against this infection and the presence of only a fragment of the spleen (*Perla & Marmorston Gottesmann* 1931) prevents the development of a manifest infection. The increase in the lymphocyte content occurred in total splenectomized rats in which development of a manifest infection was avoided by post operative treatment with tetracycline. A comparison with a tetracycline treated sham operated control material shows that the increase is not due to an effect of tetracycline on the bone marrow. It is possible, on the other hand, that the increase has been the result of a slight manifest infection which it has been possible for the animals to control by virtue of the increased lymphocyte content. The fact that subtotal splenectomy which is never followed by manifest infection did cause an even so pronounced increase is not decisive evidence against this concept, as the resistance of subtotal splenectomized animals may also be a result of their increased bone marrow lymphocyte content.

According to *Alsted* (1935) guinea pigs are never infected with *bartonella* even after total splenectomy. The present experiments therefore are aimed to elucidate whether the post splenectomy lymphocytosis of the bone marrow in the previous experiments has been caused by a secondary *bartonella* infection.

Total splenectomy was performed in 11 male guinea pigs weighing approximately 400 g. 9 guinea pigs of the same weight were sham control operated. The animals received no further treatment. They quickly recovered and gained weight satisfactorily. At the time of the bone marrow investigations 60 days after operation their mean weight was about 600 g. The technique used differs only slightly from that described by *Yoffey* (1960) for quantitative study of the bone marrow in guinea pigs. Details have been previously described (*Bierring* 1960, *Bierring & Grunnet* 1963).

Humeral bone marrow was used and smears were prepared using the serum film technique of *Harris* (1956). At least 2000 cells from each marrow suspension were differential counted and the absolute number of cells per mm^3 of marrow was calculated for each main cell group. Accessory splenic tissue was never found at autopsy of experimental animals.

The following are the mean values (± standard error) for the total number of nucleated cells and for cells in the main cell groups per mm^3 of bone marrow in splenectomized and in control animals. Total number of nucleated cells in experimental animals 1 665 083 ± 42 500 and in control animals 1 606 666 ± 114 100. The numbers of lymphocytes in experimental animals and in control animals respectively were 317 943 ± 24 500 and 244 122 ± 24 000, of erythroid cells 345 911 ± 27 500 and 440 599 ± 44 200, of myeloid cells 854 744 ± 34 400 and 769 049 ± 47 700 and of damaged cells 91 790 ± 7 450 and 109 055 ± 12 800. In addition to the cell groups mentioned the total number of nucleated cells includes cell types which are present in relatively small numbers, among these blast cells, plasma cells, reticulum cells and unidentifiable cells.

Total splenectomy in guinea pigs causes a slight—though significant—increase in the lymphocyte content of the bone marrow ($P < 0.05$). In none of the other cell groups were there significant differences between experimental animals and control animals. The lymphocyte increase is lower than that following splenectomy in rats but it supports the concept that the increase in the rats has not been caused by a secondary *bartonella* infection.

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In previous bone marrow studies on splenectomized guinea pigs *Ansell & Yoffey* (1956) found a slight—but not significant—increase in the lymphocyte content in their experiments however bone marrow was studied 40 days after splenectomy

The mean content of lymphocytes in our control animals is lower than the figures given by *Harris Herdan Ansell & Yoffey* (1954) *Yoffey* (1960) and especially by *Hudson Osmond & Roylance* (1963) for the normal guinea pig bone marrow These investigations were performed in guinea pigs weighing about 400 g and therefore presumably younger than our 600 g guinea pigs which possibly explains the difference in the lymphocyte content

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Announcement

During the VIII International Congress of Leprosy, held in Rio de Janeiro in September, 1963, Mr C I Crowther, President of the Leonard Wood Memorial for the Eradication of Leprosy, announced the James A Doull Awards They have been created to honor the memory and the significant scientific contributions of the late James A Doull, who served for 15 years as Medical Director of the Leonard Wood Memorial

The purpose of the Awards, whose donors remain anonymous, is to stimulate bacteriologists, biochemists, and others to undertake intensified investigations, including the exploitation of the newer knowledge and techniques, that the cultivation of Myco leprae and Myco muris may be achieved The inability to cultivate these organisms has been a source of frustration for bacteriologists from the time these two species of the genus Mycobacterium were discovered

The Award Committee consists of Dr R J W Rees of London, Dr Charles C Shepard of Atlanta, Georgia, and Dr John H Hanks of Baltimore, Maryland

Those desirous of obtaining more precise information regarding the Awards may communicate with

Mr *C I Crowther,*

President

Leonard Wood Memorial for the Control of Leprosy Inc

79 Madison Avenue

New York 16 New York

FROM THE DEPARTMENT OF BIOCHEMISTRY, STATENS SERUMINSTITUT, COPENHAGEN, DENMARK

POLYCYTHAEMIA PRODUCED BY CONSTRICTION OF THE RENAL ARTERY IN A RABBIT

By

POUL HANSEN

Received 24.viii.63

In cases of renal insufficiency one frequently finds an anaemia which is characterized amongst other things by the fact that the blood concentration of the erythropoiesis stimulating factor—erythropoietin—is low in proportion to the severity of the anaemia (e.g. *Naets & Heuse* 1962). On the other hand there have also been a number of descriptions of a combination of polycythaemia with renal disease. *Remmele* (1962) refers to 115 cases of this combination, amongst which were 79 verified cases of hypernephroma.

As attention has thus been concentrated on the importance of the kidney in the production of erythropoietin we have investigated the urine, plasma and renal tissue from rabbits in which experimental constriction of one renal artery had been performed (*Hansen* 1963b). During these investigations a moderate rise in haemoglobin concentration was observed in a number of the experimental animals, but more noteworthy was the occurrence of severe polycythaemia in a single rabbit. These observations are described in the present work.

MATERIAL AND METHODS

Experimental Animals

Female albino rabbits of the Statens Seruminstitut strain were used. Thin specimens were chosen, the body weight before operation being between 1450 and 2250 g. The rabbits were fed on the usual Statens Seruminstitut diet.

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Constriction of the Left Renal Artery

Clamps made from 6 × 12 × 0 7 mm silver plates which were bent into U form
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mp was placed in
38) These authors

Nembutal anaesthesia (Abbott 30 mg/kg body weight i v) When the incision was placed exactly along the linea alba the operation could be carried out almost bloodlessly The left renal artery was reached through the posterior peritoneum When the artery had been dissected free and lay emptied of blood on the arms of an open forceps the clamp was placed in position half way between the aorta and the kidney The clamp was moved cranially until the artery lay in the concavity at the base of the U clamp

Afterwards the clamp was turned through 180° about the axis of the artery

The anterior peritoneum was sutured with continuous catgut and the cutis with interrupted silk sutures Post operative therapy consisted of infusions of saline and penicillin There were no deaths in relation to the operation

During the post operative period 14 animals were killed in chloroform anaesthesia because of considerable weight loss Three animals died spontaneously Apart from occasional adhesions no evidence of peritonitis was found in these animals This group (group 2) together with a group 1 (which is not described) were not used in the present investigation

The remaining 15 rabbits (group 3) comprised the material for the present investigation These were observed for at least 35 days During the observation period the rabbits were weighed twice weekly Blood was taken for haemoglobin estimations once (or twice) weekly

The animals were killed by means of total bleeding in chloroform anaesthesia Sections were performed and gross changes in the organs were looked for

Blind Operations

Five rabbits (group 4) were operated upon in a similar manner except that the artery clamp was removed immediately after it had been placed in position during the same operation

Laboratory Tests

Haemoglobin estimations Photometry of blood diluted 280 times with 0 04 per cent ammonium hydroxide solution was carried out as previously described (*Hansen* 1963a)

Estimation of erythrocyte volume was carried out by means of the rabbits own erythrocytes labelled with Cr^{51}

Histological preparations Staining of sections of kidney tissue for demonstration of granules in juxtaglomerular cells was carried out by the *Bowie* method as modified by *Kaczmierczak* (personal communication) Staining of sections of bone marrow was carried out by means of haematoxylin eosin

RESULTS

Table 1 shows the haemoglobin concentrations in the rabbits in the 2nd–5th week after operation The rabbits were divided according to the degree of increase in haemoglobin concentration

Group 3 a comprised rabbit no 9468 in which there was a considerable increase in haemoglobin concentration The rabbits in group 3 b showed a slight increase in haemoglobin, whilst rabbits belonging to group 3 c showed no increase after 35 days The rabbits submitted to blind operation (group 4) showed a slight fall in haemoglobin in the first weeks after operation

TABLE 1

Haemoglobin and other Values of Rabbits at Various Intervals after Placement of Goldblatt Clamp on the Left Renal Artery

Group of rabbits	Number of rabbits	Haemoglobin concentration g 100 ml						Increase of weight %	Average calibre of clamp	% of body weight	
		before operation	2nd week	3th week	4th week	5th week	6th week			left kidney	right kidney
3 a (nc 9468)	1	12 3	13 2	15 3	16 3	16 1	17 5	—16	0 5	2 2	7 0
3 b	7	11 2	12 0	11 8	12 5	12 5		+17	0 59	2 9	3 3
3 c	7	12 0	12 1	12 0	11 6	11 5		+14	0 57	2 8	3 0
4 (blind)	5	11 9	10 9	11 2	11 5	11 7		+16			

If more than one haemoglobin estimation were performed in the same rabbit in the course of a week the mean value was used For further details see text

TABLE 2

Volume of Erythrocytes Determined by Cr^{51} Technique

Group of rabbits	Number of determinations	ml erythrocytes per kg body weight
3 a (9468)	1	36 4
3 b	6	15 3 ± 2 1
3 c	2	15 1
4 (blind)	4	13 4 ± 0 8
normal	4	14 4 ± 1 0

Rabbit no 9468 gradually lost weight from 1740 to 1470 g during the observation period It was otherwise in good condition After the first few days the weights of the remaining rabbits rose gradually and evenly

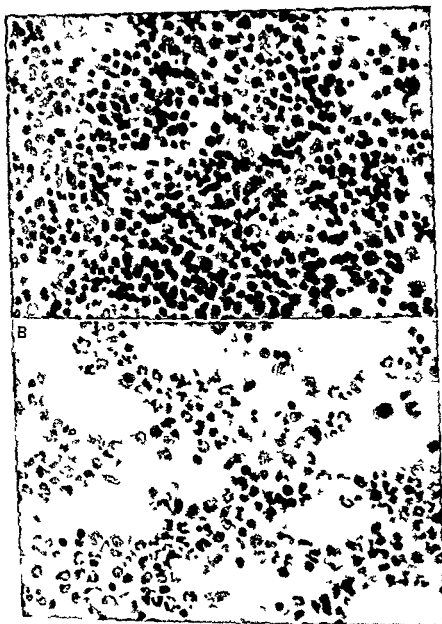
Table 2 shows the results of the estimations of erythrocyte volume carried out after the observation period The erythrocyte volume per kg body weight was considerably higher in rabbit no 9468 than in the other rabbits There was no significant difference in erythrocyte volume per kg body weight in the other operated, the blind operated, or the normal rabbits

In the course of 14 days 56 ml hypercoagulable blood could be removed from rabbits no 9468, with a resultant fall of haemoglobin concentration from 17 5 to 15 4 g/100 ml On the day it was killed the thrombocyte count in this rabbit was 360,000/mm³ The blood urea was 63 mg/100 ml, as determined by the method of *Andersen & Strange* (1959)

At section no gross changes were found in the organs, apart from shrinking of the clamped kidneys, and hypertrophy of the contralateral kidneys In particular, there was no evidence of pulmonary stasis or ascites In all cases the clamps were in position, surrounded by fibrous tissue The calibre of the clamp was measured after removal Comparison of the measurements of the clamps from groups 3 b and 3 c (see Table 1) revealed no certain relationship between the calibre of the clamp and the reaction

Fig 1 A shows the microscopic findings in bone marrow from the proximal end of the femur of rabbit 9468 For comparison bone marrow from a normal rabbit is shown (Fig 1 B) The bone marrow from rabbit 9468 is hyperplastic, with obvious dominance of erythropoiesis, whilst in the marrow from the normal rabbit granulopoiesis predominates

As it has been stated that there seems to be a relationship between the erythropoietin production and the number of granules in the juxtaglomerular cells, we have examined histological preparations of the kidney tissue from rabbit no 9468 In the clamped kidney in both afferent vessels and intralobular arteries, there was considerable hyperplasia of the media with innumerable epithelioid cells containing a large number of juxtaglomerular granules (Fig 2)



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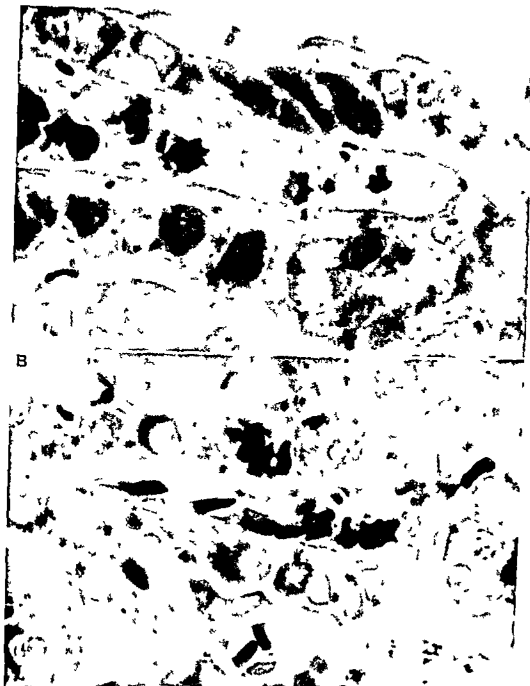


Fig 2

Microphotographs of clamped kidney from rabbit no. 9468 stained by the Bowie method (modified).—A The epithelioid cells in the media layer of an interglomerular artery (in longitudinal section) contain juxtaglomerular granules. B In an afferent vessel near the glomerulus (right) epithelioid cells containing juxtaglomerular granules scattered throughout the cytoplasm are seen (Magnification × 1400.)

In the untouched kidney only a few epithelioid cells were to be seen in the arterioles, and granules were found only occasionally

DISCUSSION

Mantz et al (1960) have shown that transient torsion of the kidney around its pedicle, so that the blood supply is interrupted, can lead to an increase of the reticulocyte count in rats After performing Goldblatt operations on rabbits *Nakao et al* (1960) were able to demonstrate an increase in the reticulocyte count, increased Fe^{59} incorporation, and increased erythropoiesis in the bone marrow

More recently it has been demonstrated that erythropoietin was present in rabbit kidneys after clamping the afferent artery, whereas no erythropoietin was demonstrable in the contralateral kidney (*Hansen* 1963b) During these experiments it was observed that one of the rabbits (no 9468), which is described in the present paper, developed a considerable polycythaemia

We feel that the polycythaemia may have developed as a result of the renal clamping The fact that only this one rabbit reacted with such a large increase in haemoglobin may be explained as the result of a fluke, in which the renal clamping was maximal, without the kidney becoming necrotic

The fact that the increase in haemoglobin concentration was not the result of a haemoconcentration was proved by estimation of the erythrocyte volume and by the observation that large volumes of blood could be removed without causing the haemoglobin concentration to fall to normal levels

No signs of heart failure with pulmonary stasis, which might explain the polycythaemia, were observed

Erythropoietin has previously been demonstrated in the urine from this rabbit (*Hansen* 1963b) This makes it reasonable to exclude the possibility that the increased haemoglobin concentration was the result of uncontrolled activity of the bone marrow

Uncontrolled bleeding during or following the operation necessary to place the clamp on the renal artery could stimulate an increased rate of erythropoiesis as measured by an increase in the reticulocyte count or in Fe^{59} incorporation but it could not give rise to an increase in the erythrocyte volume or haemoglobin concentration We therefore consider the observation of the development of such pronounced polycythaemia after constriction of one renal artery to be definite evidence of the ability of the clamped kidney to produce erythropoietin

It has been stated that there seems to be a relationship between the erythropoietin production and the number of granules in the juxtaglomerular cells in the kidneys (*Hirashima & Takaku* 1963 *Golfarb & Tobian* 1963) In the case of rabbit no 9468 we have been able to confirm the presence of increased granularity in the juxtaglomerular

cells of the clamped kidney In the untouched kidney juxtaglomerular granules were difficult to detect This observation obviously provides no evidence of a causative connection between the increased granularity of the juxtaglomerular cells and the production of erythropoietin

SUMMARY

A description is given of an experiment in which the left renal arteries of 32 rabbits were constricted In some cases a slight increase in haemoglobin concentration was observed, whilst one of the rabbits developed a pronounced polycythaemia, with hyperplasia of the bone marrow The juxtaglomerular granularity was much more pronounced in the clamped kidney from this rabbit than in the untouched kidney

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FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY, GENERAL HOSPITAL MALMO SWEDEN

MUCOCELE AND CARCINOMA OF THE APPENDIX

By

SVERKER HELLSTEN

Received 14 ix 63

Mucocele of the appendix consists of a more or less distended part of the appendix containing mucus The condition was first described by *Rokitansky* (1842) Mucocele of the appendix is rare In a large series of appendectomies, it was found with a frequency of 0 3 per cent (*Woodruff & McDonald* 1940) and in autopsy series in 0 2 per cent (*Castle* 1915) In an autopsy series from Malmo consisting of about 6 700 autopsies during the years 1957–1962, the frequency of mucocele was 0 19 per cent

The clinical picture is usually not really typical Some patients, however, report recurrent attacks of pain in the right iliac fossa, where a lump sometimes can be felt (*Sachs & Hoffmann* 1951) Treatment usually consists of appendectomy If malignant changes are suspected or proved, the operation is usually extended to include ileocaecal resection

Most cases of mucocele of the appendix are due to stenosis secondary to acute or chronic inflammation of the appendix Of other causes, mention might be made of different forms of tumour, such as carcinoid of the appendix and caecal cancer (*Hilsabeck et al* 1952) which may cause partial or complete obstruction of the proximal part of the appendix Mucocele of the appendix has also been described in the absence of obstruction (*Fahr* 1940)

The microscopic picture of mucocele of the appendix varies with the stage of development of the lesion (*Carleton* 1955, *Sluiter* 1960) Thus, the glands may be *dilated* owing to increased intraluminal pressure Papillary structures of hypertrophic epithelium are somet...

...y represent different stages of a single condition or different forms of mucocele, a benign type and a malignant tumour

An important though rare, complication of mucocele of the appendix is peritoneal pseudomyxoma, in which mucus escapes and initiates a chronic productive peritonitis, possibly with proliferation of mucus-forming epithelium on the peritoneal surfaces with accumulation of mucus in the abdominal cavity (*Roulet* 1938 *Diezel* 1949, *Schreiber* 1955)

Peritoneal, pseudomyxoma behaves clinically like a malignant tumour, and it has been discussed whether the condition should not be regarded as a highly differentiated, mucinous adenocarcinoma. Opinions differ widely on this point. Below, some cases are described in which the histological picture may contribute to elucidate this question. In 3 out of 4 cases the histological picture was clearly carcinomatous, while the fourth showed no reliable evidence of infiltrative growth.

CASE REPORTS

Case 1. F. J. No. 3977/61.

A man, aged 56, had been operated upon for gastric ulcer in 1925. He had been treated with pneumothorax in 1945–1953 because of tuberculosis of the right lung. In 1956 he was admitted to the surgical Department, Malmo General Hospital, because of pain persisting for about 1 year, in the right iliac fossa. Double phase contrast examination of the colon and cholecystography revealed nothing of interest. Urography and retrograde pyelography showed left sided hydronephrosis of obscure nature and the same degree of dilatation at urography about 2 years later. In 1958 he had dyspepsia, at this time roentgen examination of the stomach revealed deformation of the bulbus because of ulcer.

In May 1961 the patient was again admitted to the Surgical Department because of abdominal pain, persisting for 3 days in the right lower quadrant. Surgical ex
d that the right iliac fossa was
through a perforation about the
as thick as a thumb. Since histological examination gave reason to suspect mucinous adenocarcinoma the patient
onths later 'Second look', however, revealed
the operation thrombosis developed in the
ith antiprothrombin. Roentgen examination of the colon revealed a polyp in the sigmoid but otherwise nothing of interest.

The operative specimen consisted of a piece of a cyst containing mucinous masses and ruptured at the top. Histologically, the wall of the appendix was found to consist of hyaline connective tissue containing rather abundant round cell foci. The surface facing the lumen was found to be partly covered by a layer of atypical cylindric epithelial cells often with basal nuclei. In some parts the epithelium was exuberant with a tendency to papillary projections and the epithelial cells often contained mitoses (Fig. 1). The outer surface of the appendix showed some areas of hyperplastic epithelium. The intestinal lumen contained amorphous pale acidophilic masses of mucus. Centrally in the intestinal wall bands of partly preserved smooth
some areas lymph follicles and plasma cell infiltrates. The
of amorphous pale acidophilic material con
leucocytes and round cells but no epithelial
cells. Epithelial prolifera
appendix were so advanced and atypical that mucinous adenocarcinoma in the ruptured mucocele was suspected.

Case 2. S. A. No. 6636/61.

A 64 year old woman who had previously been healthy was admitted in August 1961 to the Department of Surgery, Malmo, because of abdominal pain and diarrhoea which had persisted for 3 days. The patient's clinical appearance was strongly suggestive of peritonitis and surgical exploration was decided upon because of suspected perforated appendicitis. Operation revealed a hard lump behind the caecal pole and a small perforation at the base of appendix. The retrocaecal infiltrate which was believed to be an old appendiceal abscess and the appendix were removed. Post
at the site of the wound. Histological examination
noma and about 1 month later the patient was
resection with side to side anastomosis between
the ileum a
operative specimen showed no signs of cancer.
Nor did the mesenterial lymph nodes studied. Apart from a postoperative wound abscess nothing remarkable was observed during convalescence nor at a later follow up.



Figs 1 2

Fig 1 (Case 1) Small papillary formations with atypical mucocele epithelium No infiltrative growth Htx eosin × 75

Fig 2 (Case 2 Typical appearance of mucocele with exuberant markedly folded atypical epithelium and abundant mucus Htx eosin × 75

Peritoneal, pseudomyxoma behaves clinically like a malignant tumour, and it has been discussed whether the condition should not be regarded as a highly differentiated, mucinous adenocarcinoma Opinions differ widely on this point Below, some cases are described in which the histological picture may contribute to elucidate this question In 3 out of 4 cases the histological picture was clearly carcinomatous, while the fourth showed no reliable evidence of infiltrative growth

CASE REPORTS

Case 1 F J No 3977/61

A man aged 56 had been operated upon for gastric ulcer in 1925 He had been treated with pneumothorax in 1945–1953 because of tuberculosis of the right lung In 1956 he was admitted to the surgical Department Malmo General Hospital because of pain, persisting for about 1 year, in the right iliac fossa Double phase contrast examination of the colon and cholecystography revealed nothing of interest Urography and retrograde pyelography showed left sided hydronephrosis of obscure nature and the same degree of dilatation at urography about 2 years later In 1959 he had dyspepsia, at this time roentgen examination of the stomach revealed deformation of the bulbus because of ulcer

In May 1961 the patient was again admitted to the Surgical Department because of abdominal pain, persisting for 3 days in the right lower quadrant Surgical exploration because of assumed appendicitis revealed that the right iliac fossa was filled with a gelatinous mass Similar fluid escaped through a perforation about the width of a finger, in the appendix which was about as thick as a thumb Since histological examination gave reason to suspect mucinous adenocarcinoma the patient
onths later "Second look", however revealed
the operation thrombosis developed in the
ith antiprothrombin Roentgen examination
of the colon revealed a polyp in the sigmoid but otherwise nothing of interest

The operative specimen consisted of a piece of a cyst containing mucinous masses and ruptured at the top Histologically the wall of the appendix was found to consist of hyaline connective tissue containing rather abundant round cell foci The surface facing the lumen was found to be partly covered by a layer of atypical cylindric epithelial cells often with basal nuclei In some parts the epithelium was exuberant with a tendency to papillary projections and the epithelial cells often contained mitoses (Fig 1) The outer surface of the appendix showed some areas of
hyperplastic
masses of mu
muscle were
mucus from the peritoneum consisted of amorphous pale acidophilic material containing scattered polymorphonuclear leucocytes and round cells but no epithelial cells Epithelial proliferations in the appendix were so advanced and atypical that mucinous adenocarcinoma in the ruptured mucocele was suspected

Case 2 S A No 6636/61

A 64 year old woman who had previously been healthy was admitted in August 1961 to the Department of Surgery Malmo because of abdominal pain and diarrhoea which had persisted for 3 days The patient s clinical appearance was strongly suggestive of peritonitis and surgical exploration was decided upon because of sus
perforated appendicitis Operation revealed a hard lump behind the caecal

noma and about 1 month later the
resection with side to side anastomosis between
e operative specimen showed no signs of cancer
Nor did the mesenterial lym ides studied Apart from a postoperative wound abscess nothing remarkable was observed during convalescence nor at a later follow up

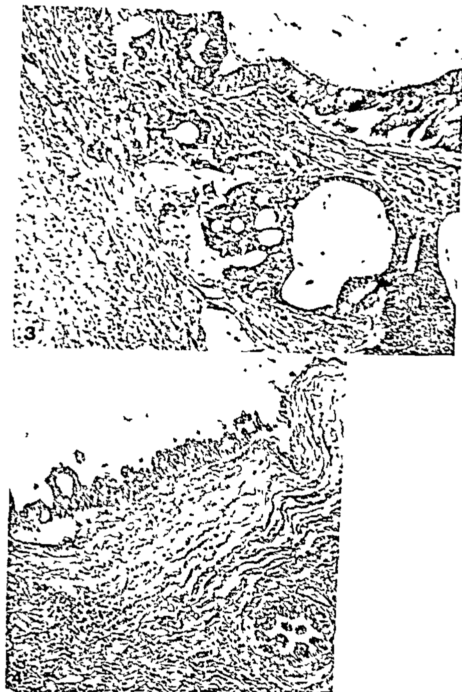




Figs 5–6

Fig 5 (Case 3) Glandular tubular formations of very atypical cylindrical epithelium infiltrating wall of mucocele Htx eosin × 75

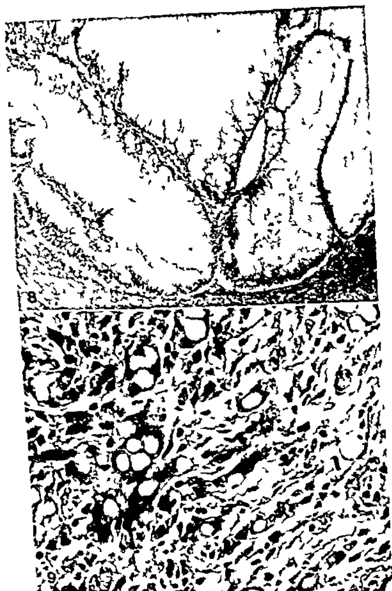
Fig 6 (Case 3) Mucocele with mucinous cylindrical epithelium showing considerable polymorphism with irregular layers of nuclei of different sizes Htx eosin × 680



Figs 3–4

Fig 3 (Case 2) Infiltrative growth of markedly atypical mucocele epithelium Htx-eosin × 120

Fig 4 (Case 3). Mucocele epithelium with small papillary formations on inner wall of appendix and infiltrative growth of a very atypical epithelium of same appearance in deeper layers of the wall Htx eosin × 75



Figs 8 9

Fig 8 (Case 4 Abundant mucin production from a very atyp cal mucocele ep the lium Htx-eosin × 75

Fig 9 (Case 4) Diffuse infiltrati n of very atypical muc nous epithel um into the muscularis propria The cancer cells are d screte or cr wded in very atypical glandular tubules Htx eosin × 480

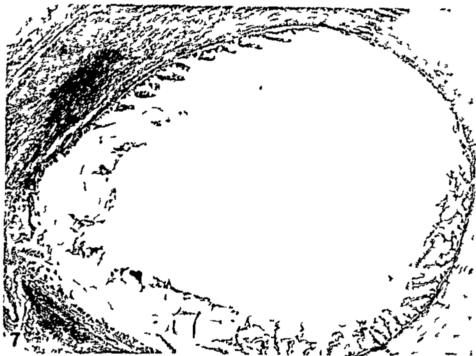


Fig 7

(Case 4) Mucocele with markedly folded mucinous papillary epithelium formations on inner side Htx eosin × 15

Specimens from the first operation contained an appendix with a rounded cavity surrounded by indurated and partly haemorrhagic tissue At the base of the appendix there was a small perforation The lumen of the appendix contained numerous usually calcified masses Large areas of the lumen had no epithelial lining and the surface consisted of granulation tissue partly covered with fibrin Within small areas a mucous membrane with some irregular glands was seen In some areas the epithelium was pale and mucinous and partly basophilic and atypical In several other areas the mucous membrane was very exuberant with papillary projections lined by markedly mucus forming cylindrical epithelium of the type usually occurring in mucocele (Fig 2) In other areas however the epithelium was more basophilic with crowded irregularly arranged nuclei and contained numerous mitoses In a few areas distinct growth of adenocarcinoma with polymorphous epithelium was seen Atypical glandular tubules showed incipient infiltration in the muscularis propria (Fig 3) The tumour was mucinous The intestinal wall contained foci and bands of inflammatory cells and was partly loose and oedematous The subserous fatty tissue was fibrous and showed a marked inflammatory reaction No tumour growth was seen outside the lumen of the intestine The lumen contained scattered calcifications and in some areas fragments of bone tissue Foreign body giant cells were seen in the granulation tissue

Case 3 H L No 1032/62

A woman aged 78 with a 10 years history of colic reported that since July 1962 attacks had become more frequent In the middle of September she was admitted to the Surgical Clinic in Malmo because of acute cholecystitis She was then febrile and jaundiced On the 12th of October the patient suddenly died

Autopsy revealed *multiple obstructing pulmonary emboli* One stone was found

duct The appendix adherent t the

7 cm it was markedly distended and

d some pale yell w green gelatinous

appendix was bserved

of *Sluiter* the histological changes do not justify a diagnosis of cancer This conception is based, among other things, on the fact that infiltrative growth and metastases are though to be absent in appendiceal mucoceles, and he does not accept a few earlier published cases with metastases (*Roulet* 1938, *Diezel* 1949)

Three out of the four present cases showed infiltrative growth of mucinous adenocarcinoma in the wall of the appendix No doubt, *Sluiter's* conception may therefore be refuted, which is also apparent from an article by *Wilson* (1962), who described 17 cases of primary cancer of the appendix Five of these were of this type and are called malignant mucoceles, while 5 were cancers of the appendix largely presenting the same histological picture as adenocarcinoma of the colon The remaining cases could not be assigned with certainty to either of the 2 groups, some showed the histological features of both groups It therefore appeared justified to conclude that neither group can be distinguished as an entity, and that the varying picture of the cancer of the appendix may be regarded as a manifestation of a higher or lower degree of differentiation Spread of cancers of the appendix of mucocele type to the abdominal cavity may be per continuitatem, in contrast to adenocarcinoma of the appendix of colon type the lymphatics do not appear to be involved (*Uhlein & McDonald* 1943, *Hilsabeck* 1953)

Microscopic examination of mucoceles occasinoally reveals epithelium-lined cavities, situated in the wall of the mucocele, filled with mucus, and in open communication with the lumen of the appendix These cavities are believed to be due to protrusion of the mucosa caused by the increased intraluminal pressure owing to the production of mucus (*Sluiter* 1960) Similar changes were also observed in some of the present cases Sometimes the walls of the cavities are lined by atypical cylindrical epithelium, sometimes by more or less well-preserved groups of epithelial cells floating freely in the masses of mucus In one of the cases (No 3) a large number of serial sections were studied, and it was found that some of the minor or major and very atypical elements of the [illegible]

wi [illegible]

at[illegible] [illegible] in the muscularis propria were found to be less differentiated and to infiltrate more diffusely in the form of foci and streaks of mucus secreting cells (*Fig* 9) These observations argue against the hypothesis that the glandular tubular elements [illegible] – the mus-

[illegible] uggestive

[illegible] the risk

[illegible] pseudomyxoma As observed in one of the present cases (No 3), *perforation* of the wall of mucocele can occur even in the absence of structure of the [illegible]

Histologically the appendix showed a markedly thickned wall with infiltrates of round cells and leucocytes many of which were eosinophilic On the inner side a mucinous epithelium with papillary structures was seen which showed considerable atypia with basophilia and large dark basal nuclei (Fig 4) The epithelium was very exuberant in some parts distinctly infiltrating the muscularis propria (Figs 5 ... ts with a number of ... well preserved frag ... roscopic perforation ... rved in the serosal surface No metastases could be seen in sections from the regional lymph nodes in the mesenterium

Case 4 S.J No 4625/61

A 52 year old woman who had hitherto ... of Obstestrics and Gynaecology in Malmo ... ing during the menopause Curettage sho ... suspected transition to adenocarcinoma The patient was submitted to extirpation of the uterus at which the appendix was also removed The tip of the appendix was filled with large masses of mucus and contiguous parts of the appendix were obliterated Histological examination revealed mucinous adenocarcinoma with mucocele of the appendix The patient was therefore referred to the Department of Surgery Malmo where ileocaecal resection was done Microscopical examination revealed no residual cancer in the mesenterial lymph nodes and in the operative specimen After the operation diabetes mellitus was diagnosed and treated with insulin In addition the N P N gradually increased the urinary excretion decreased and the body temperature gradually rose The last few days the patient had diarrhoea and passed into a state of shock death occurred some days later in the Department for Renal Diseases in Lund Autopsy revealed tubular nephritis In addition purulent tracheobronchitis and bronchopneumonia were diagnosed No residual cancer was seen

The appendix removed at the first operation was markedly changed The most proximal part was of normal gross appearance A segment 2 cm long was obliterated and showed a yellow white cut surface The tip of the appendix consisted of large masses of mucus which in some areas were porly encapsulated Histological examination revealed large amounts of mucus in the distended distal part of the ...

areas mucus had escaped through the wall and appeared in the subserous fatty tissue The obliterated part of the appendix showed connective tissue at the site of the mucosa However numerous foci with strands of atypical glandular tubules built of mucus secreting cells were seen These grew into the submucosa and partly out into the muscularis but did not appear to penetrate the whole wall of the appendix (Fig 9)

DISCUSSION

Cases of mucocele with cytological changes resembling those seen in preinvasive cancer have been described (*McCollum* 1951 *Wilson* 1962) American investigators (*Woodruff & McDonald* 1940 *Hilsabeck* 1953 *Woolner* 1953) assert that mucocele of the appendix may occur in a benign as well as a malignant form and that the latter may develop from the former On rupture of the wall of the appendix the malignant type can cause peritoneal pseudomyxoma The American conception has been rejected by *Sluiter* (1960) who challenges the term malignant mucocele He regards the occasional proliferative papillary formations lined by atypical epithelium in a mucocele as manifestations of the regenerative power of the appendical epithelium In the opinion

FROM THE DEPARTMENT OF PATHOLOGY CENTRAL HOSPITAL
VÄNERSBORG SWEDEN (HEAD O GRÖNTOFT)

"MUCOCELE APPENDICIS" WITH PSEUDOMYXOMA PERITONEI AND PULMONARY METASTASES

By

THORBJÖRN BERGE

Received 14 ix 63

A point on which opinions still differ is whether pseudomyxoma peritonei is due to growth of mucus secreting cancer of the abdominal cavity Below, a case is described in which the malignant nature of the mucus secreting epitehilum was evident from pulmonary metastases

CASE REPORT

In 1959 a man aged 59 sought medical advice because of 2 or 3 years diffuse abdominal pain anorexia and loss of bodyweight Explorative surgery because of suspected malignant disease revealed a dilated appendix which had apparently ruptured and no v l ...

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... type as before
The patient deteriorated and died 3 months after the operation *Post mortem* examination (501/62) showed the same picture of the abdomen as that seen at operation In addition all the lobes of both lungs were of firm elastic consistency and contained disseminated indurations the size of rice seed Metastases were suspected but careful examination revealed no primary tumour other than the one in the appendix Histological examination of the lungs however showed numerous large cystic spaces filled with mucinous fluid and lined by the same pale mucus secreting epithelium as that described above (Fig 2)

DISCUSSION

Many authors, as *e g Boyd* (1961) *Robbins* (1962) and *Will* ... (1960 ...
believe that ...
toneı Oth ...
ever that ...
that obstr ... atrophy of the epithelium and accumulation of only moderate amounts of mucinous fluid in the abdomen

SUMMARY

Four cases of mucocele of the appendix with perforation and escape of mucus through the wall of the appendix are described Atypical papillary epithelium was seen in the mucocele in all 4 cases, in 3 of the cases the wall of the appendix was found to be infiltrated by cancer The investigation confirmed the assumption that mucocele of the appendix may be due to mucinous adenocarcinoma Such cases of mucocele indicate *radical surgical treatment*

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ADDENDUM

Case 1 Two years after second look operation the patient got tired with anemia Laparotomy was performed and the peritoneal cavity contained jellylike substance Histological examination showed mucous masses and strands of mucinous epithelium of the same type as in the appendiceal mucocele Consequently the disease had progressed to a typical pseudomyxoma peritonei (highly differentiated mucinous carcinoma)



Fig 3
Lung Mucin secreting epithelium (van Gieson × 192)

besides that the mucinous fluid contains no epithelium and that the condition is cured by appendectomy, after which the mucous exudate disappears

According to *Boyd* (1961) the large production of mucus in pseudo myxoma peritonei may be due to a metaplasia of the epithelium to that of coelomic type *Novak* (1959) compares the condition with mucinous cystoma of the ovaries and suggests the possibility of a teratoma in which the mucus producing epithelium has suppressed the other com ponents

In experimental investigations on rabbits (*Bergan* 1950) in which the appendix with intact circulation, was opened and thereby exposed to the free abdominal cavity, mucus was found to form although the epithelium did not proliferate beyond its normal site On auto transplantation by which the appendix was divided into small pieces and left in the abdominal cavity, no epithelium grew He concluded The occurrence of the mucinous masses is not caused by epithelial cells outside the appendix"

In acute inflammation the appendix may rupture or its distal part may be severed whereby epithelial cells may escape into the abdominal cavity Epithelial cells are disseminated following spontaneous and traumatic ruptures or in association with surgery of the digestive tract, but they never give rise to pseudomyxoma peritonei

Slater (1960) is of the opinion that the increased intraluminal pressure in obstructive mucocele, stimulates proliferation of the epithelium



Figs 1 2

Fig 1 Appendix Several papillary structures in mucosa of appendix and mucus filled epithelial proliferations in wall (van Gieson × 80)

Fig 2 Lung Lumina filled with mucinous fluid and surrounded by mucin secreting epithelium (McManus × 75)

FROM THE DEPARTMENT OF PATHOLOGY ULLEVÅL HOSPITAL OSLO

THE EFFECT OF CORTISONE ON THE LIPID PATTERN AND THE CYTOLOGY OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

By

KRISTEN ARNESEN

Received 25 ix 63

This paper deals with certain aspects of the so called spontaneous adrenocortical lipid depletion in mice of the AKR/O strain and derived hybrid lines (*Arnesen* 1955 1956 1963)

The lipid depletion is determined by the single recessive adreno cortical lipid depletion gene whose effect is in some way dependent upon the sex hormones

Metcalf (1960) confirmed the existence of the spontaneous adreno cortical lipid depletion and claimed that it is the expression of a state of hypofunction

From cytological evidence (*Arnesen* 1963) supported by a previous electron microscopic study (*Molbert* & *Arnesen* 1960) it seems that the lipid depleted cells of the adrenal cortex (especially the *zona fasciculata*) are very active at least in terms of basic metabolic functions This interpretation is in accordance with the studies of *Symington Duguid* & *Davidson* (1956) The evidence at hand however does not permit any conclusions as to the specific endocrine function of the lipid depleted mouse adrenal cortex of the AKR/O strain

High doses of ACTH restore the lipid pattern of adult AKR/O mice to a roughly normal level (*Arnesen* 1956) This might indicate that the hypophysis of the AKR/O strain was hypofunctional with respect to the adrenocorticotrophic hormone Definite conclusions were not drawn be cause of the large doses of ACTH necessary to give a distinct effect

In the present study the problem has been attacked from another angle Through a feed back mechanism the administration of cortisone will inhibit the adrenocorticotrophic activity of the hypophysis If the spontaneous adrenocortical lipid depletion reflects a pituitary hypo function cortisone treatment will result in a further depletion of the cortical lipids if possible

The work has been supported by Grosserer N A Stangs legat til kreftsykdommers bekjempelse

But experimental ligation of the appendix (*Grodinsky & Rubnitz* 1941) has failed to produce epithelial proliferation and hence *Sluiter*'s theory does not appear convincing

The finding of mucin-secreting epithelium in the muscular layer of the appendix need not be due to increased intraluminal pressure, as such epithelium has also been found in cases in which the proximal part remained continuous with the distal part (*Fahr* 1940) In addition, careful histological examination will often reveal areas of cellular atypia as reported by *Hellsten* (1964) on page 473 of this number

All these observations argue against the possibility of a ruptured obstructive mucocele as a cause of pseudomyxoma peritonei The marked power of proliferation of the epithelium suggests malignancy, a highly differentiated mucin-secreting cancer of low biological malignancy which first grows *per continuitatem*

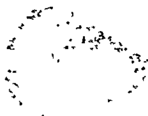
The present case, however, showed that this type of cancer can also give rise to remote metastases This was described as early as in 1938 by *Roulet*, in this case, which was well documented with photographic reproductions, secondary growths were found in the intercostal musculature

SUMMARY

A case of mucocele-like, highly differentiated mucin secreting cancer of the appendix with pseudomyxoma peritonei and lung metastases is described In true pseudomyxoma peritonei the mucinous masses always contain proliferating epithelium In ruptured obstructive mucocele only mucinous fluid occurs in the abdominal cavity and the condition can be remedied by appendectomy The term mucocele appendicis should be reserved for the latter type

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1



2

Figs 1-2

Fig 1 AC 340 56 days old Untreated Adrenal gland Spontaneous cortical lipid depletion Sudan × 34
Fig 2 AC 337 52 days old Cortisone for 3 days Adrenal gland Cortex refilled with lipid Sudan × 34

The animals were killed on the 3rd 6th day of the experiment
Usually one adrenal gland from each animal was fixed in 4 per cent formaline emb[illegible]

[illegible] ...ing carried out to the point where the mitochondria were clearly demonstrated

RESULTS

At autopsy the adrenal glands of the cortisone-treated animals presented an altered macroscopical appearance The colour of the glands *in situ* was distinctly yellow, instead of the deep red which characterizes the spontaneously lipid-depleted adrenals of the untreated AKR/O and AC male mice

In all experimental series the Sudan-stained frozen sections from the

On the other hand, the adrenocortical lipid depletion might be the expression of an increased pituitary—adrenocortical activity with a high production rate and little or no storage of hormones and/or precursors. If this is so, the depression of the pituitary activity through cortisone will tend to slow down hormone production and enhance the storage, which would eventually result in a reaccumulation of lipids in the adrenal cortex.

To test these alternative hypotheses, four series of experiments have been carried out, where adult, male AC mice were treated with cortisone, after which they were killed and the lipid pattern and cytological structure of the adrenal cortex studied.

MATERIAL AND METHODS

Male mice of the AC hybrid line were used. This line is derived from the AKR O strain and the mice are homozygous for the adrenocortical lipid depletion.

The experimental conditions are tabulated in Table 1. The preparation CORTONE (cortisone acetate) from the firm Merck Sharp & Dohme was given undiluted in the 1st, 2nd and 4th series of experiment (1 ml = 25 mg). In the 3rd series it was diluted 1:10 in saline. The amount injected intramuscularly *pro dosi* was 0.1 ml (in the 1st series even 0.05 ml).

TABLE 1

Cortisone Experiments. AC Males. Age in Days. Daily Intramuscular Dose of Cortisone Acetate in mg. Animals Killed at ×

	Age in days	Day of experiment					
		1st	2nd	3rd	4th	5th	6th
1st series							
AC 84	117	2.5	2.5	1.25	1.25	×	
AC 85	87	2.5	2.5	1.25	1.25	×	
AC 86	88	2.5	2.5	1.25	1.25	1.25	×
2nd series							
AC 162	105	2.5	2.5	2.5	2.5	×	
AC 163	125	2.5	2.5	2.5	2.5	×	
AC 164	138	2.5	2.5	2.5	2.5	×	
AC 165	108	2.5	2.5	2.5	2.5	×	
AC 166	116	Control with distilled water				×	
3rd series							
AC 177	78	0.25	0.25	0.25	0.25	×	
AC 178	78	0.25	0.25	0.25	0.25	×	
AC 179	146	0.25	0.25	0.25	0.25	×	
AC 180	140	0.25	0.25	0.25	0.25	×	
AC 181	154	0.25	0.25	0.25	0.25	×	
AC 182	154	Control with saline				×	
4th series							
AC 334	172	2.5	2.5	2.5	×		
AC 335	197	2.5	2.5	2.5	2.5	×	
AC 336	115	2.5	2.5	2.5	2.5	×	
AC 337	52	2.5	2.5	2.5	×		
AC 338	52	Control untreated					
AC 339	194	2.5	2.5	×			
AC 340	56	Control untreated					

during three days to an AC male and cannot be distinguished from the appearance in normal mouse strains

The cytological details of the adrenal cortex in control and cortisone treated AC males are presented in Figs 3 and 4 respectively

The appearance of the cortical cells (*zona fasciculata*) is identical in the controls (Fig 3) and in the previously reported mice with spontaneous adrenocortical lipid depletion (*Arnesen* 1963 Figs 5 and 7) In the cortisone treated mice (Fig 4) the structure of the cortical cells does not differ from that in animals with the adrenocortical lipid pattern demonstrable in most strains of mice (*ibidem*, (Figs 6 and 8)

The results were comaparable in all of the four experimental series

DISCUSSION

It appears from these experiments that the administration of cortisone within the given dose range results in a re accumulation of lipids in the adrenal glands with spontaneous cortical lipid depletion This change in lipid pattern is accompanied by a profound alteration in the structure of the cytoplasm the numerous mitochondria being apparently replaced by lipid vacuoles

Presupposing that cortisone acts through a depression of the adrenocorticotrophic activity of the hypophysis the results reported might indicate that in the AC (and AKRO) mice the spontaneous adrenocortical lipid depletion and the associated cytological structure of the *fasciculata* cells are signs of a hyperactive pituitary stimulation of the adrenal cortex rather than the opposite

This tentative conclusion is in agreement with a previous discussion of the functional meaning of the adrenocortical lipid depletion as regards the adrenal cortex itself (*Arnesen* 1963) Neither of these studies support the hypothesis that the spontaneous adrenocortical lipid depletion signifies a state of pituitary and/or adrenocortical hypofunction

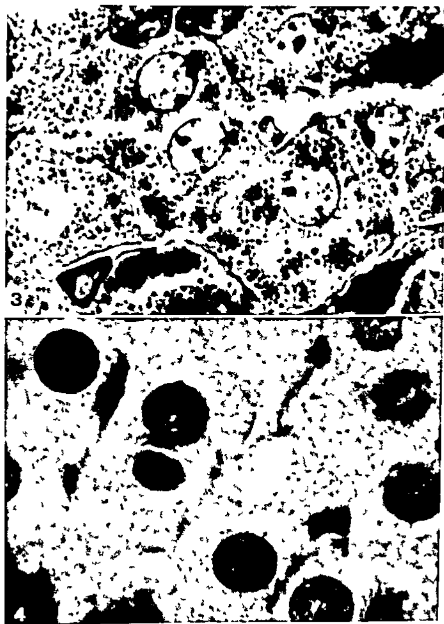
One should be very careful however in making definite conclusions in so far as the dose of cortisone is probably far outside the physiological range An effect of cortisone differing from the one presupposed in this study can not be ruled out either

The problem of the functional significance of the spontaneous adrenocortical lipid depletion has not been solved so far and further research along different lines is in progress

SUMMARY

In mice with spontaneous adrenocortical lipid depletion treatment with cortisone reverses the lipid pattern and the cytological details of the cortical (*fasciculata*) cells to conditions found in most normal strains of mice

Functionally the spontaneous adrenocortical lipid depletion may be



Figs 3 4

Fig 3 AC 338 52 days old Untreated Cells in z. *fasciculata* filled with numerous large mitochondria Iron haematoxylin × 2400

Fig 4 AC 337 52 days old Cortisone for 3 days Cells in z. *fasciculata* with vacuolated cytoplasm and indistinct mitochondria Iron haematoxylin × 2400

cortisone-treated animals demonstrated a more or less complete reversal of the adrenocortical lipid pattern to the normal type with an even and dense distribution of sudanophilic material throughout the whole cortex

The normally lipid-depleted adrenal cortex of adult, healthy and untreated AC (and AKR/O) males is depicted in Fig 1 The pattern demonstrated in Fig 2 results from the administration of 7.5 mg of CORTONE

UNIVERSITY INSTITUTE OF BIOCHEMISTRY (CHIEF PROFESSOR F LUNDQUIST PH D) AND RHEUMATIC RESEARCH LABORATORY, UNIVERSITY INSTITUTE OF PATHOLOGICAL ANATOMY (CHIEF PROFESSOR G TEILUM M D), COPENHAGEN, DENMARK

PARAPROTEINS AND ACID MUCOPOLYSACCHARIDES IN PRIMARY AMYLOIDOSIS

Biochemical and Histologic Studies of Four Human Cases of Primary Amyloidosis

By

J CLAUSEN and H E CHRISTENSEN

Received 1 x 63

The metabolism of immuno globulins seems related to the metabolism of serum glycoproteins Thus hyper-immuno-globulinaemia is often associated with an increase in sero mucoid and alpha 1–(S 3,5) glycoprotein (*Heiskell Carpenter, Weimer & Nakagava* 1961) However, while the immuno globulins are formed by plasmacells, the glycoprotein production seems dependent upon metabolism of the reticulo endothelial (RE) system demonstrated by the fact that glycoprotein deposits in secondary amyloidosis are related to function of RE-cells (*Teilum* 1956) Furthermore in rheumatoid arthritis, the hyper-immuno-globulinaemia, can be associated with secondary amyloidosis, probably caused by local liberation of glycoproteins in the tissue (*Teilum* 1952) Serum-glycoproteins seem to be formed by liberation from the connective tissue (*Schultze & Heide* 1960) The connective tissue seems to be built up of proteins and acid mucopolysaccharides (Chondroitin-sulphates, hyaluronic acid kerato sulphate, and probably traces of heparin) (*Gibian* 1959) Acid mucopolysaccharides contain carbohydrates also present in ot

pr tempting to correlate dys- and

bo acid mucopolysaccharide meta-

nunication, data will be given concerning the relationship between primary amyloidosis, paraproteinaemia, and deposits of hyaluronic acid in heart tissue

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These investigations have been supported by the *Danish League Against Multiple Sclerosis The Danish League Against Rheumatism* and by a gift from *Thomas B Thriges Fund* Odense Denmark

Gratitude is expressed to *professor K Brochner M*

ment A professor *A Tybjærg Hanse*

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tal and finally *chief physician K*

Denmark for the materials used in the investigations

the result of increased pituitary stimulation of the adrenal cortex. Definite conclusions in this respect, however, are not yet possible.

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c) a rabbit antiserum against normal human gamma globulin was produced as described above The gamma globulin used was isolated by preparative electrophoresis (*Muller Eberhard* 1960) in Pevicon 870 By cutting out the 3 last cms of the cathode patterns which after a running time at about 20 hours Elution with saline gave an immunologic pure gamma globulin The extract was concentrated by vacuum dialysis to 5 per cent W/V

d) a rabbit antiserum against normal human beta 2 A (gamma 1 A) globulin (From Behringwerke Marburg Lahn Germany) This antiserum was absorbed in the proportion 1 10 with the above mentioned gamma globulin thus giving rise to an antiserum strictly specific against beta 2 A (gamma 1 A) globulin

by

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solution (*Foster & Pearce* 1961)

Infrared analysis of the acid mucopolysaccharides was performed in an Unicam SP 200 spectrophotometer as described by *Clausen & Andersen* (1963) *Clausen & Hansen* (1963) All the infrared analyses were performed by the potassium bromide disk technique

Isolation of the Acid Mucopolysaccharides from Heart Tissue

Normal heart tissue as well as heart tissue from case T S G (10 gram) were deep frozen to 25° C in an X press homogenizer (*Edebo* 1961) The deep freezing was performed by dipping the X press containing the heart tissue into —25° C cold ethan l cooled with CO ice Afterwards when the temperature —25° C was obtained the tissue was exposed to 2000 tons pressure by pressing the deep frozen tissue through a 1 mm hole at 25 atm Under these circumstances the ice changed its structure *from ice type I to type III* a process characterised by a volume reduction of about 20 per cent (*Edebo* 1961) Hereby a thick homogeneous suspension was obtained After a thorough blending with half a volume tap water a supernatant extract was obtained by centrifugation at 18 000 g The clear supernatant was

s ubin was estimated by direct photometry at 422 mμ

(*Goldberg* 1961)

Th

0 20 25 40 40–50 50–75 per

extract The precipitates were dia

followed by dialysis for 24 hours

against saline Afterwards the fractions of acid mucopolysaccharides (as Na salts) could be isolated by evaporation on watch glass heated from below with steam Dryness was obtained by storage *in a* vacuum exсicator over silicagel The fractions were now ready for blending with potassium bromide (4 mg 100 mg KBr) in a Unicam Ball Mill After a thorough blending for 5 minutes pellets were made by pressing in Unicam s evacuable die (MkIII) The KBr pellets thus obtained containing the acid mucopolysaccharide fractions were investigated by infrared analysis as described by *Clausen & Andersen* (1963) and *Clausen & Hansen* (1963)

RESULTS

At autopsy cases 1 and 2 showed enlargement and stiffness of the heart (weights resp 620 and 510 grs) (Fig 1) In case 3 macroglossia was present

Histological examination The diagnosis of amyloidosis was con-

Primary amyloidosis is a rather seldom occurring disease It can be hereditary or not hereditary (*Rukavina, Block, Jackson, Fallis, Carey & Curtis* 1956, *Frederiksen, Gotzsche, Harboe, Kiær & Mellemgaard* 1962) A localized form of primary amyloidosis, affecting only the heart has been described in old persons, but otherwise it is characterized by systemic deposits throughout the body, especially in the tongue, skin heart, striated muscles, blood vessels, and the kidney

The clinical manifestations can be heart insufficiency, renal failure, and secondary broncho-pneumonia and other complaints based upon pathophysiological changes caused by amyloid deposits

MATERIAL AND METHODS

Patient Material

Four patients with primary amyloidosis were object for histological and biochemical investigations

1) Case T G S (No 1186) b 20 9 19 hospitalized at Med Departm A Rigshospitalet Copenhagen (Concerning a detailed description of the clinical symptoms see *Videbæl & Drusholm* (1963))

2) Case F I J (No 3650) b 18/4 04 hospitalized at Med Departm B Rigshospitalet Copenhagen

3) Case N P N (No 3650) hospitalized at Med Department Municipal Hosp Haderslev

4) *Case N P (No 2138), b 16/3 82 hospitalized at Med Departm A The Military* Hosp Copenhagen (Concerning a detailed description of the clinical feature see *Danielsen & Clausen* (1963))

During the hospitalization blod samples from a cubital vein were taken for elec*trophoretic investigations (vide infra)*

The above mentioned patients were investigated by histological examinations of different organs Case 1 2 and 3 came to autopsy whereas the patient in case 4 is still alive Especially the heart and tongue musculature were object for histological *examinations*

Furthermore the heart tissue from case T G S was investigated biochemically for presence of paraprotein deposits and deposits of acid mucopolysaccharides

Histological Procedure and Staining Methods

[illegible] owing [illegible] green [illegible] *Mowry* 1958) Congo red and [illegible]) was used to estimate the specificity of the staining procedure for acid mucopolysaccha[illegible] ions were [illegible] destroy [illegible] Sections were treated with [illegible]

Biochemical Methods

Immuno electrophoresis was performed as described by *Grabar & Williams* 1953 modified as a micro method by *Scheidegger* 1955 The immuno electrophoreses were developed with the following antisera

a) a horse antiserum *against normal human serum* (No 511 *from the Pasteur* Institute)

b) a goat antiserum against normal human serum This antiserum was produced by injecting 0 2 ml normal pooled human serum subcutaneously in the goat every third week for 2 months *The first injection was accompanied with subcutaneous* injections of 0 5 ml complete Freund s adjuvans DIFCO Exactly 8 days after the last injection the blood was taken from a cubital vein





Fig. 2

Myocardium from case 2. The black areas show a positive amyloid reaction with Lugol's iodine solution plus sulphuric acid.

placing them or causing atrophy of these. Sometimes amyloid was found focally in large masses. The vessels in nearly all material examined often showed thickening of the walls by amyloid substance.

The amyloid showed a positive Congo red reaction but was only slightly metachromatic with methylviolet. It was slightly fuchsinophilic in the van Gieson stain. A positive reaction was found with the periodic acid Schiff procedure, however of a rather low staining intensity. In general the amyloid substance did not show metachromasia with toluidine blue or azur A. A positive reaction was found with Alcian Blue staining and in the Hale technique. Especially a positive Alcian Blue coloration was found in the periphery of each individual amyloid deposit. As the latter reaction could be prevented by treatment of the sections with streptococcal hyaluronidase this experiment was regarded to be a confirmation of the biochemical determination of hyaluronic



Fig 1

The enlarged heart from case 2 viewed from in front The heart has been opened in the ordinary way but portions of the anterior wall of the right and left ventricle in addition was removed The thickened walls of the right and left ventricles and of the septum ventriculorum are demonstrated Sections from these sites showed a positive amyloid reaction (Fig 2) The pericardium and endocardium were also thickened

firmed in all four cases In case 1 amyloid was found principally in the heart and tongue (Fig 2), but also in the lungs and in the vessels of many other organs Unfortunately the skin was not examined In case 2 amyloid was found principally in the heart, tongue, skin, and lungs, but in addition widespread in the vessels of other organs Case 3 showed amyloid substance in the tungue

Case 4 This patient is alive Biopsy of the liver has shown severe amyloidosis, whereas biopsies of the tongue and colon were negative

In the three cases, which came to autopsy, the amyloid substance had the appearance typical for primary amyloidosis The location in the heart and the tongue was interstitially between the muscle cells, re-

acid in the amyloid deposits as described below However neuramini dase treatment prior to staining with Alcian Blue also caused the dis appearance of a part of the positive reaction

Biochemical Reactions

Figs 3 6 demonstrate the immuno electrophoretic findings In all four cases the gamma globulin precipitation line deviates in the inter mediate gamma area toward the antibody through as a sign of an in creased amount of gamma antigen in a narrow interval of mobility This excess will cause the place for the optimal proportion between the an t gen and the antibody to be displaced toward the antibody reservoir Therefore the gamma globulin precipitation arc is nearer the antibody trough in the paraprotein area Thus a paraprotein (M component of gamma type) occurs in all four cases The paraprotein has an inter mediate γ mobility Furthermore it is seen that the precipitation lines for the slow and the fast part of the gamma line as well as for the l ta 2 A (gamma 1 A) globulin and beta 2 M (gamma 1 M) globulin are shortened in the anode cathode directions and longer from the anti body reservoir than normally Therefore the paraproteinaemia in these cases is associated with a hypo immunoglobulinaemia Finally it has to be mentioned that case E I J seems more complicated than the other ones because of the presence of another paraprotein with beta 1 mobility (Fig 4) In the cathodic part of the concavity of the trans ferrin precipitation arc a sharply deviating abnormal bow can be seen This precipitation bow seems to cross the gamma globulin preci pitation line and furthermore the beta 2 A (gamma 1 A) precipitation line cannot be seen Therefore it is tempting to identify the beta 1 para protein as being of the beta 2 A (gamma 1 A) type But in this case no

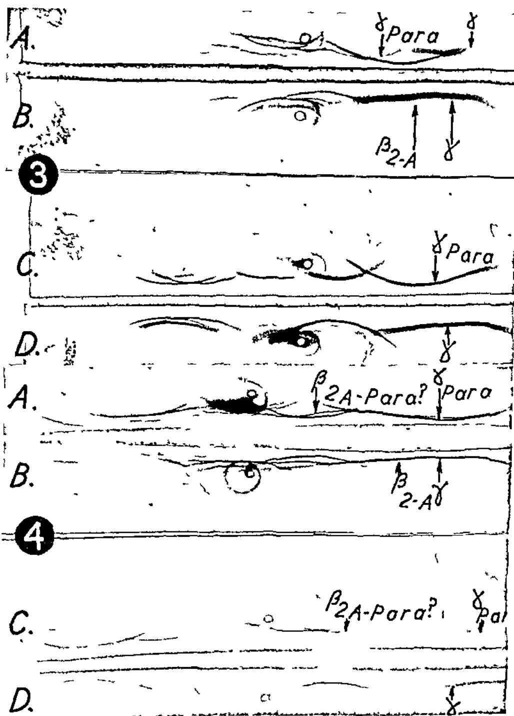
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F g •

F gs 3 4

F g

sc ts a γ gl bulin paraprotein —B Immuno electrophoresis of un l luted normal human serum (The same as A but the serum is diluted 1 4 with sal ne —D The same as B l ut the serum is d luted 1 4 with saline



A.
γ
Para
γ
B.
β 2-A
γ
3
C.
γ
Para
D.
γ
A.
β 2A-Para?
γ
Para
B.
β 2-A
γ
4
C.
β 2A-Para?
γ
D.
γ

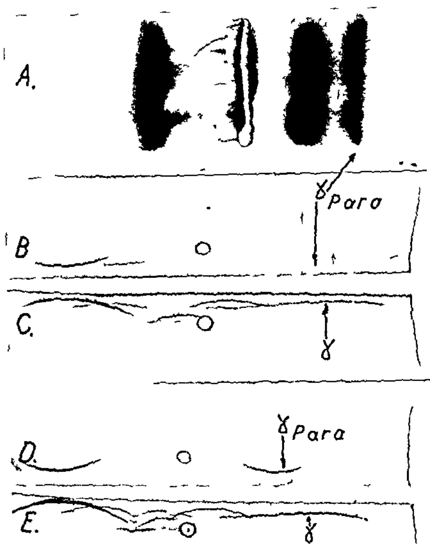
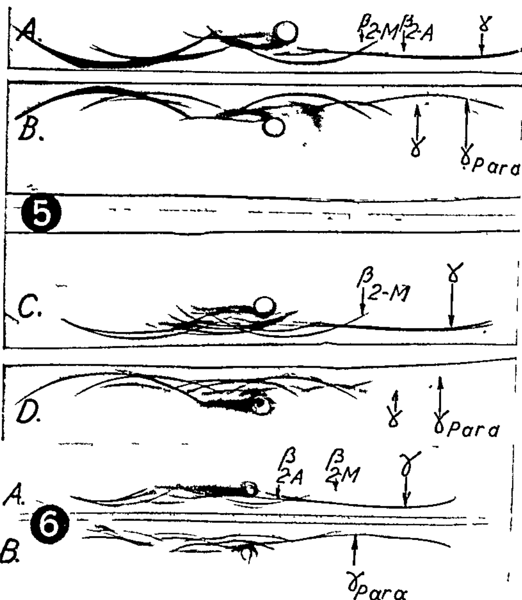


Fig 7a

A Agar gel m cr electrophoresis of the extrable proteins of the heart from patient T S G A el n m ... heart ext ... o electrophoresis of the ... bow can be seen C Immun ... B and C are developed with a g ... e as C —D and E are devel ped with a horse antiserum

serum has been available after death for further examination and identification of the protein with strictly specific beta 2 A antiserum

Fig 7a and b show the immuno electrophoretic findings of extractable proteins from the heart (case T G S) Apart from the preci pitation lines corresponding to albumin and transferrin an accentuated



Figs 5–6

Fig 5 Immuno electrophoresis of serum from patient (N P) with primary amyloidosis Above the immuno electrophoresis is developed with a goat anti

re
m
io
in

precipitation bow can be seen —C The same as A D The same as B

Fig 6 Immuno electrophoresis of serum from patient (N P N) with primary amyloidosis The immuno electrophoresis is developed with a horse antiserum against normal pooled human serum —A Immuno electrophoresis of undiluted normal human serum B Immuno electrophoresis of undiluted serum from N P N The deflecting γ paraprotein bow can be seen

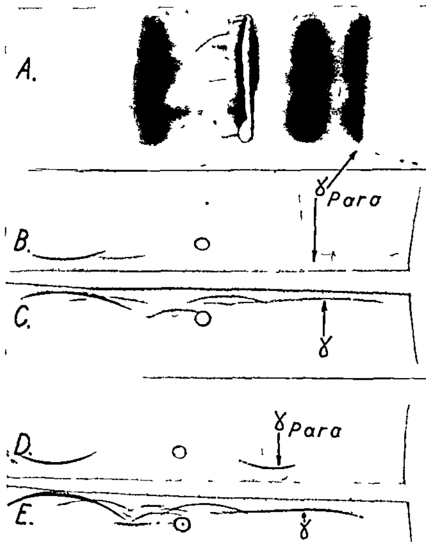


Fig 7a

A Agar gel micro electrophoresis of the extrable proteins of the heart from patient T S G A slow moving γ fraction can be seen —B Immuno electrophoresis of the heart extract from T G S The γ paraprotein precipitation bow can be seen —C Immuno-electroph resis of undiluted normal human serum —B and C are developed with a g at antiserum D The same as B —F The same as C —D and F are devel ped with a horse antiserum

serum has been available after death for further examination and identification of the protein with strictly specific beta 2-A antiserum

Fig 7a and b show the immuno electrophoretic findings of extractable proteins from the heart (case T G S) Apart from the preci pitation lines corresponding to albumin and transferrin an accentuated

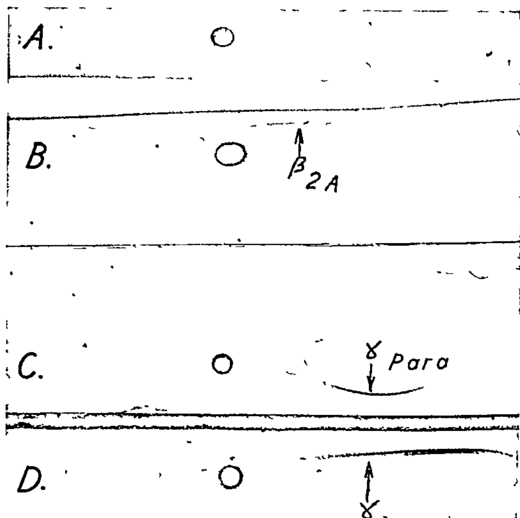


Fig 7b

A Immuno electrophoresis of extractable proteins of the heart from T G S developed with a rabbit antiserum against β 2 A No precipitation line can be seen corresponding to this protein – B Immuno electrophoresis of undiluted normal human serum developed as A —C The heart extract of T G S developed immuno electrophoretically with an antiserum against human γ globulin The deflecting γ para protein precipitation bow can be seen —D Undiluted normal human serum developed as C

deviating precipitation line with intermediate gamma-mobility occurs This line can be developed with antiserum against gamma globulin, but not with anti-beta-2-A (gamma-1-A)-globulin Thus the paraprotein is present in the pathological heart tissue extract, but not in normal heart tissue The heart extracts revealed in electrophoresis albumin and beta-, gamma protein fractions, Fig 7c), but no distinct α-1- or α-2-fractions thus admixture of blood seems of minor significance corresponding to the Sephadex filtration which revealed 0 134 gram hemoglobin per 100 ml extract The paraprotein band can be seen in the agar-gel micro-electrophoresis of the heart extract (T G S)

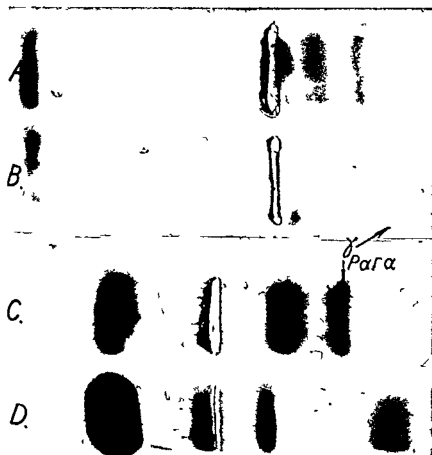


Fig 7c

A Agar gel m .. ..

Fig 8 and 9 demonstrates the acid mucopolysaccharide pattern in the heart tissue (case T G S) A slow moving main fraction, migrating cathodically for the chondroitin sulphate, can be seen Anodically of this main fraction a very faint diffuse fraction can be seen The fractions stained distinctly with Alcian Blue, indicate these to be acid mucopolysaccharides The cathodic one seems predominant This fraction could not be demonstrated in normal heart extract

Fig 10 ›

able •

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... with 40–50 per cent and 50 75 per cent ethanol Group frequences can be seen for both of them at 3400, 2900, 1660 1640,

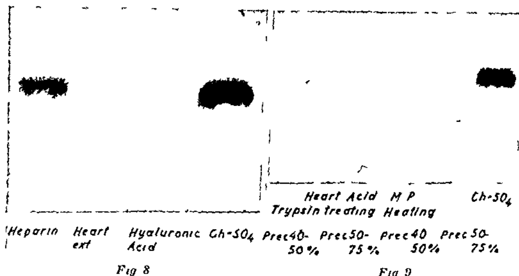


Fig 8   *Fig 9*

Fig 8 Paper-electrophoresis in veronal buffer (pH = 8 6 ionic strength μ = 0 05) of heart extracts from case *T G S* stained with *Alcian Blue* for acid mucopolysaccharides The patterns are correlated to those of Heparin depolymerized hyaluronic acid and chondroitin sulphate

Fig 9 Paper-electrophoresis in Lithium chloride solution (pH = 2 8 ionic strength μ = 0 2) of acid mucopolysaccharides isolated by alcohol (ethanol) fractionation after enzymatic digestion of proteins in heart extract from case T G S Two fractions of acid mucopolysaccharides could be obtained in intervals between 40 to 75 per cent ethanol

1560, 1420, 1385, 1320, 1050–1160, 920, 880, 850, 700 cm^{-1} No band could be seen at 1250 cm^{-1} When these are correlated to those of chondroitin sulphate (A + B) heparin and hyaluronic acid and, furthermore, correlated to results already published (see the survey by *Gibian* 1959 and *Clausen & Andersen* 1963) it is possible to obtain the following conclusions The absorption band at 3400 cm^{-1} can be explained by the stretching vibrations of the bonded OH groups as well as by bonded and free NH groups The 2900 cm^{-1} band can have its origin in the stretching vibrations of CH_2 and CH_3 groups The band at 1660 1640 cm^{-1} can be explained by the *C = O stretching vibration for instance* in carboxyl groups, but also mono-substituted amide groups can give rise to an absorption at 1650 cm^{-1} as well as at 1560 cm^{-1} Most probably the 1560 cm^{-1} band can be attributed to N-acetyl groups The bands at 1420 and 1385 cm^{-1} can be caused by the presence of CH_3CO- (bending in plane, scissor) The absorption band at 1320 cm^{-1} can be attributed

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Fig 10

Infrared analysis of fractions of acid mucopolysaccharides isolated by ethanol fractionation of heart extract from case T G S

The spectograms are correlated to those obtained from standard preparations of chondroitin sulphate and hyaluronic acid Absorption bands can be seen at 3400 2900 1720, 1650, 1420, 1160 1100–1000 880 850 cm^{-1} similar to those of partial depolymerized hyaluronic acid

Chondro t n Sulphate
Hya uron c ac d
Ac d MP from heart t ssue
(Case GTS) (50% C_2H_5OH)
Pr mary Amylo dos s
Hyaluron c Ac d
(Part al depolymer zed)
5000
4000
3000
2000
1800
1600
1400
1200
1000
800
650

Fig 10

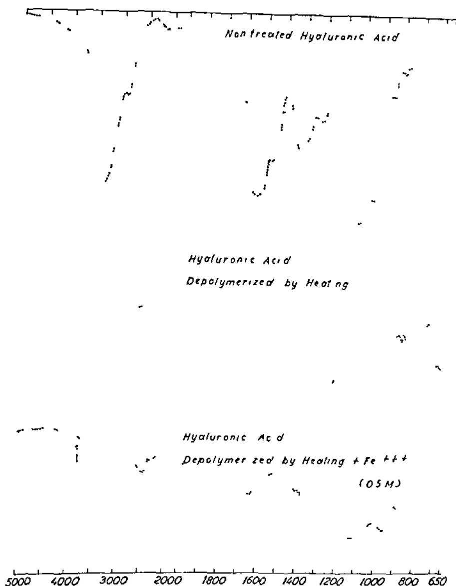


Fig 11

Infrared spectograms of untreated hyaluronic acid and hyaluronic acid altered by steam heating for three quarters of an hour in saline solution (0 5 per cent) with or without Fe^{++} It is seen that by treatment one or two distinct absorption bands appear in the finger print area (the area below 1000 cm^{-1}) of the hyaluronic acid IR spectrogram The absorption bands are at 880 and 850 cm^{-1}

to the OH bending in plane On the other hand the S = O stretching vibration in sulphate groups at 1240 cannot be found The great absorption band about 1040 1160 cm^{-1} can be seen in all carbohydrates and can be explained as caused by C-O-stretching vibration as well as by C-C vibrations and finally by CN vibrations

Thus above 1000 cm^{-1} the group frequences of the two unknown acid mucopolysaccharides seem identical with those of a sulphate free acid mucopolysaccharide which can be stained with Alcian Blue Below 1000

cm⁻¹ both preparations reveal two outstanding absorption bands at 880 and 850 cm⁻¹ which do not occur in any of the standard acid mucopolysaccharides

However, a similar spectrum could be obtained by dissolving a standard hyaluronic acid in saline (1 per cent) and by evaporating the water again, according to the isolation procedure, used in this communication (Fig 11) These finger print absorption bands of treated hyaluronic acid must reveal chemical changes These can also be seen in the paper-electrophoretic pattern Thus, non-treated hyaluronic acid gives rise to a non migrating Alcian Blue positive fraction on paper-electrophoresis at pH = 2 8, but after heating the solution, especially if iron ions are present, the hyaluronic acid will give rise to two faster moving fractions with a mobility somewhat slower than that of chondroitin sulphate (*Clausen, Dyggve & Melchior* 1963) The two fractions obtainable by alcohol fractionation between 40–50 and 50–75 per cent ethanol revealed identical absorption frequences Only the proportion between the absorptions at 880 and 850 cm⁻¹ was not identical, probably revealing different proportions between the above-mentioned two fractions (subunits of hyaluronic acid with different mobility in paper electrophoresis)

DISCUSSION

Previously it was found that primary and secondary amyloidosis exhibited similar serum protein changes (*Calkins & Cohen* 1959) These results seem, however, in discrepancy with the data in this communication where paraproteinaemia was associated with primary amyloidosis (vide infra) while as demonstrated secondary amyloidosis can be encountered in allergic dysproteinaemic conditions (*Teilum* 1948)

Paraproteinaemia seems to occur predominantly in myeloma and Waldenstrom's macroglobulinaemia (*Heremans, Laurell, Mårtensson, Heremans Laurell, Sjoquist & Waldenstrom* 1961, *Drivsholm & Clausen* 1963) However paraproteinaemia has been described, occurring associated with other diseases, but extremely seldom Thus it has been described in chronic lymphatic leucemia, in cancer of the digestive system tuberculosis [illegible] seminatus (*Heremans* 1960, [illegible] *ns, Laurell, Sjoquist, Walder* [illegible]

The [illegible] imary amylo [illegible]

[illegible] electrophoresis revealed in one case the paraprotein to be deposited in the heart musculature, thus explaining the heart complaint of the patient

The heart also contained deposits, which in paper-electrophoresis could be stained with Alcian Blue and which possessed a mobility slower than that of chondroitin sulphate, but faster than non treated

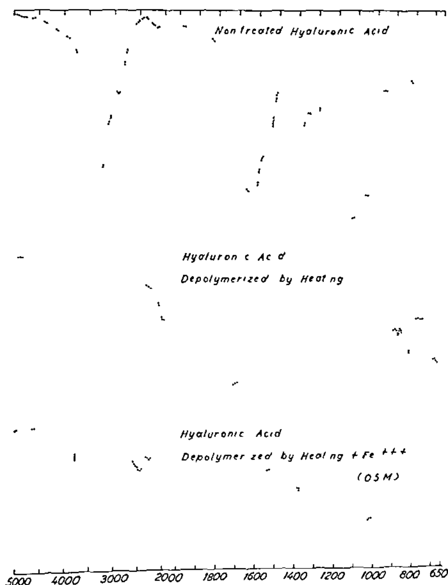


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Thus above 1000 cm^{-1} the group frequences of the two unknown acid mucopolysaccharides seem identical with those of a sulphate free acid mucopolysaccharide which can be stained with Alcian Blue Below 1000

cent Bence-Jones proteinuria was absent in all cases Although myeloma has been reported to be complicated with amyloidosis and although sometimes the distribution of the amyloid was found similar to that in paramyloidosis (*Bayrd & Bennet* 1950 *le Coulant David Chausse & Texier* 1953 *Osserman* 1959 *Fruhling Kempf & Chadli* 1962), this possibility of a hidden myelomatous disease is very unreasonable in the present cases Also the plasmocytosis in the bone marrow in some cases of primary amyloidosis (*Teilum* 1948 *Propp Scharfman Beebe & Wright* 1954 *Conn & Sundberg* 1961) was absent in our cases

The investigations reported here show principal differences between the composition of the amyloid substance in the main types of amyloidosis Thus the proteins stored in human and experimental amyloidosis of secondary type are identical with the glycoproteins of the serum (*Larsen* 1957) and the acid mucopolysaccharide is a sulphated acid mucopolysaccharide (see *Kennedy* 1962) In this paper the proteins of primary amyloidosis accumulations were shown to consist of paraproteins in one case and the acid mucopolysaccharide was identified as hyaluronic acid In this connection it shall be mentioned that *Braunstein & Buerger* (1959) by histological methods found that amyloid in myeloma cases did not contain much acid mucopolysaccharide

The problems of pathogenesis will not be discussed here Readers are referred to (*Teilum* 1964)

SUMMARY

Four cases of human primary amyloidosis were the object for histologic and biochemical investigations Autopsy was performed on three of the cases Amyloid deposits were found in the heart tongue and other organs The amyloid was histochemically shown to be stainable with Alcian Blue especially in the periphery of the deposits The stainability with Alcian Blue disappeared after treatment with bacterial hyaluronidase These findings were in agreement with the biochemical investigations of the heart in one of the cases Thus paper electrophoresis of the heart extract stained with Alcian Blue for acid mucopolysaccharides revealed a fraction with a mobility slower than chondroitin sulphate and heparin This fraction was isolated and identified by means of infrared analysis to be sulphate free The finger print area of the infrared spectrum contained absorption frequences similar to those of altered hyaluronic acid Immuno electrophoresis revealed in all four cases γ-paraproteinaemia and the serum from one of the cases contained furthermore another paraprotein The heart which was investigated for accumulations of acid mucopolysaccharides was also investigated for proteins by means of agar gel microelectrophoresis and immunoelectrophoresis The paraprotein occurring in serum could also be demonstrated in the heart tissue The results have been discussed on the basis of the literature

hyaluronic acid (commercially available). By infrared analysis the deposits were found to give group frequencies as an acid mucopolysaccharide, but differed in the finger print area from hyaluronic acid as well as from chondroitin sulphate (A + B) and heparin. The group frequencies indicated the substance to be sulphate free. By treating the commercially available hyaluronic acid, as the acid mucopolysaccharide from the heart tissue under the isolation procedure, the finger print absorption frequences were identical in the two preparations. Two fractions of acid mucopoloysaccharides could be isolated by ethanol fractionation, one precipitating at 40–50 per cent and one between 50–70 per cent ethanol. But these fractions gave identical infrared spectra (see Table 1). Only the proportion between the two absorption bands at 880 and 850 cm⁻¹ was different. This can probably be explained by a splitting of the hyaluronic acid in at least two subunits. This is supported by the fact that paper-electrophoresis of commercially available hyaluronic acid, treated as described above, gives rise to two fast moving bands (*Clausen, Dyggve & Melchior* 1963).

TABLE 1

Absorption Frequencies in the Infrared Area Specific for Different Acid Mucopolysaccharides. The Area below 1 000 cm⁻¹ Is Called the Finger Print Area.

Differences in the IR spectra of Acid Mucopolysaccharides

Area cm⁻¹	1200–1380	1000–900	900–800	800–700
Heparin	1240	980–928	850	780
Chondroitin sulphate A	1240	928	852	725
Chondroitin sulphate B	1240	928	855–840	712
Chondroitin sulphate B	1240	1000	820	775
Hyaluronic Acid				
Hyaluronic Acid (depolym)			880–850	

Also the histologic investigations of the heart tissue (cases 1 and 2) revealed that the Alcian Blue stainability of the amyloid was prevented by streptococcal hyaluronidase, the finding of which is indicative for the presence of hyaluronic acid in the amyloid substance. The presence of siliac acid in amyloid organs was demonstrated by *Klenk & Faillard* 1955, *Diezel & Pfleiderer* 1959, and *Schmitz-Moormann* 1961. The experiments with neuraminidase in this paper are confirmatory to these findings.

In none of the cases the clinical history of the patients revealed any preceding disease, which might be held responsible for the development of amyloidosis of secondary type. The cases were then regarded as primary amyloidosis, which is also in accordance with the ordinary organ distribution of amyloid in paramyloidosis (cases 1–3).

Clinical examination, including haematological examination of bone marrow and X-ray examination of bones, did not show any signs of myelomatosis. The highest differential count of plasma cells was 4 per

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FROM THE DEPARTMENTS OF HISTOLOGY AND PATHOLOGY,
UNIVERSITY OF GOTHENBURG, SWEDEN

LIVER CHANGES IN CEROID STORAGE DISEASE IN CHILDHOOD

A Correlated Light and Electron Microscopic Study

By

SOREN BJORKERUD and ULF SCHELLIN

Received 29 viii 63

Since the end of the 19th century it has been known that the human liver contains pigmented, intracellular granules—called lipofuscin granules—in amounts increasing with age. A fairly reliable idea of their light microscopic appearance and histochemical properties has been acquired over subsequent decades, and, more recently, data on their electron microscopic characteristics have been published. Yet it remains unknown how these lipofuscin granules are produced and which function, if any, they have in the cell (cf *Bjorkerud* 1963)

The fact that large amounts of lipofuscin-like granules are found in the liver cells in children with ceroid storage disease—a disorder of which only a few cases have been reported (*Oppenheimer & Andrews* 1959)—suggests that the quantity of lipofuscin granules accumulated is not related exclusively to age. Moreover, it is possible to induce accumulation of *lipofuscin-like granules in liver cells of animals in* experimental nutritional deficiency cirrhosis (*Lillie et al* 1941, *Endicott & Lillie* 1944). Similar changes have also been observed after intravenous fat administration (*Neglia et al* 1963) and in hepatic injuries following glucagon perfusion (*Ashford & Porter* 1962)

The present study on the liver tissue in two cases of ceroid storage disease was undertaken to elucidate the intracellular changes associated with that condition and to enable comparisons with previously reported lesions of liver tissue *in advanced age and in experimentally induced* accumulation of pigment granules

MATERIAL AND METHODS

The material consisted of biopsy specimens obtained at laparatomy from two livers one from a girl aged 3 years (Case 1) and one from a male infant aged 6 months (Case 2), in both of whom ceroid storage disease was suspected[1]

[1] A full clinical report of the two cases will be published elsewhere

Light Microscopy

The tissue was fixed in 10 per cent neutral formalin solution. Frozen sections were used for staining with Sudan III, Sudan IV and Oil Red O (*vide infra*) and paraffin embedded sections for other staining procedures. Long Ziehl-Neelsen, periodic acid [illegible]

Examination by fluorescence microscopy was done on deparaffined sections mounted in immersion oil or glycerol.

Electron Microscopy

The [illegible] cent solution and sectioned [illegible] post stained [illegible] 1960).

RESULTS

Light Microscopy

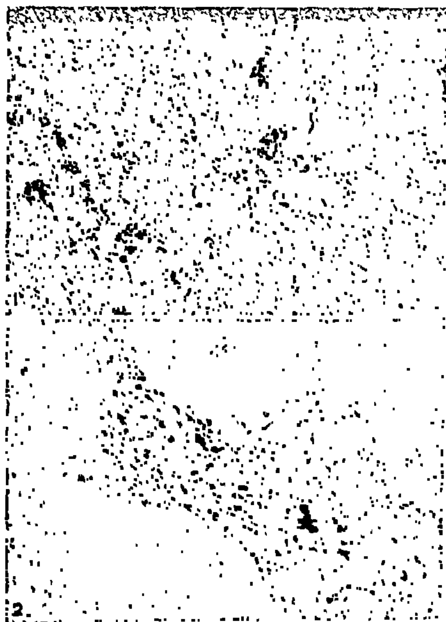
The liver of Case 1 exhibited slight fatty degeneration in the parenchymal cells and a mild increase of the periportal connective tissue. The parenchyma contained scattered granuloma-like formations with macrophages loaded with Sudan black B positive material (Fig. 1). These cells showed in unstained sections accumulations of dark yellowish brown particles. Pigment granules of similar hue were present also in the Kupffer cells. Moreover, the cells in non-granulomatous portions of the parenchyma contained deposits of light yellowish brown pigment granules.

The liver of Case 2 displayed more advanced changes with pronounced portal cirrhosis in which coarse connective tissue strands split up the parenchyma into irregular lobules (Fig. 2). Slight fatty degeneration was apparent in the remaining parenchyma. Unstained sections showed that portions transformed into connective tissue contained abundant deposits of dark yellowish brown pigment granules, whereas parenchymal cells contained a sparse amount of lighter yellowish brown granules.

In both cases the paler pigment granules showed light yellowish brown fluorescence and the darker ones a less intense and darker yellowish brown fluorescence in ultraviolet light (Figs. 3 and 4). Both varieties of pigment granules, also in both cases, yielded positive reactions with Sudan black B, performic acid-Schiff, periodic acid Schiff, Schmorl's ferric ferricyanide and long Ziehl-Neelsen.

Electron Microscopy

Sections of liver tissue from the female patient had a well preserved structure with some fat droplets in the parenchymal cells. At various sites granuloma like formations were found with clusters of cells containing abundant deposits of osmiophilic granules (Fig. 5). Moreover,



Figs 1–2

Fig 1.

Fig 2

granules of similar appearance occurred in Kuppfer cells and parenchymal cells, especially in the neighbourhood of bile canaliculi (Figs 5 and 7).

Marked alterations of the liver structure were found in the second case; coarse connective tissue strands splitting up the parenchyma into small irregular lobules in which the cells often had an irregularly di-



F gs 3 4

F g 3 Autofluorescent p gment granules in parenchymal macrophages from Case 1 Fluorescence phot micrograph × 440

F g 4 Autofluorescent pigment granules in connect ve t ssue from Case 2 Flu re s ence photom crograph × 440

lated endoplasmic reticulum The osmiophilic granules predominated in reg ons transferred into connective tissue (Fig 6) but occurred also in parencl ymal cells usually those near bile canaliculi

The osmiophilic granules varied widely in size and shape (Figs 7 11) They were of the following main types

1 (Fig 7) Granules with a limiting membrane and containing more or less extensive vacuolar regions



Fig 5

Survey electron micrograph of liver from Case 1. In the upper portion parenchymal cells containing scattered lipid droplets (LD) and groups of pigment granules (P) are seen. In the lower part a granuloma-like focus is apparent with abundant pigment granules deposited in the cells. Occasional liver cells (LC) are interspersed among the pigment-containing cells. × 3400

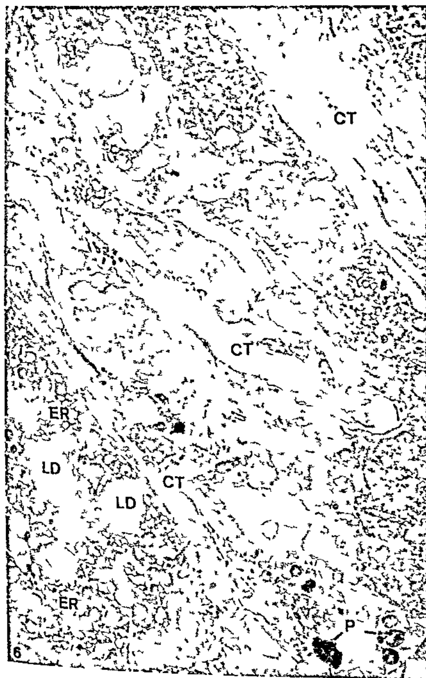


Fig 6

Sur y electron micrograph of liver fr m Case 2 The parenchyma is traversed by coarse connect ve tissue trabeculae (CT) in which there are macr phages containing p gment granules (P) The liver cells contain l pid droplets (LD) and a dilated end plasm c reticulum (ER) × 5900

P
P
P
BC



F g 10

Conne t e t s ue ma ophage h p gm nt g anules of t pe III composed ma nl) f osm oj h c partl les and str ped area × 46 000

II (F g 8) Cranules l ke those of type I but in ad l tion containing constituent strongly osmioj hilic particles of varying size in large num bers

III (Figs 9 11) Granules exhibiting constituent osmioj hilic j articles and in a ldition homogenous grey reg ons and strij ed areas

In granules of tyj es II and III a varying proportion of the osmioj h lic part cles showe l ultrastructural features similar to ferritin (Fig 11)

In l oth cases the granules deposited in the cells of the liver paren

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F gs 7 9

F g 7 L er cell b rde ng n a l c canal cu us BC and conta ning p gment gra n e f typ I (P wh h ha e a l m t ng membrane and acuole like areas 14 000

F g 8 A j gment g anule of type II w th large numbers of osm oph l c part cles × 67 000

F g 9 Kupffer c ll w th a p gment g anule of type III compo ed of const tuent o m oph l j art cles hom genous g y reg ons and str ped areas × 51 000

P
P
P
P
BC

cirrhosis (cf *Pearse* 1960). It is suggested that the osmiophilic granules on the electron microscopic level represent lipofuscin granules. This inference is based upon comparisons between the occurrence and distribution of the osmiophilic granules in the electron micrographs and lipofuscin granules in the light microscope. Its validity is strengthened by the fact that isolated lipofuscin granules show strongly osmiophilic regions in the electron micrographs (*Bjorkerud* 1963). Classified in accordance with Pearse's scale for rating lipofuscin granules by their maturity, the granules observed in granulomas, Kupffer cells and connective tissue regions are more mature than those in parenchymal cells. The electron microscopic classification of osmiophilic granules presented under Results is based on comparisons between their light microscopic location and maturity and the electron microscopic location and appearance. Accordingly, pigment granules of types I, II and III on the electron microscopic level represent increasing maturity of the granules as assessed by light microscopic criteria. Formations resembling pigment granules of types I and II have previously been reported in the liver by various workers including *Essner & Novikoff* (1960) who classified them as lysosomes. Granules of type III have also been described in the liver (*Essner & Novikoff* 1960) and in a variety of other tissues (cf *Bjorkerud* 1963). They are usually known as lipofuscin granules.

Deposition in the liver of pigment granules resembling those described in the present study has been induced experimentally. After intravenous fat administration (*Neglia et al* 1963) the pigment is probably of exogenous origin from the lipids administered and located mainly in clusters of macrophages (granulomas). In hepatic injury following glucagon perfusion (*Ashford & Porter* 1962) it has been proposed that the source of the lipid might be membraneous material surrounding cell organelles. The similarity of the changes described in this study and those observed after intravenous administration of fat suggests that the same causative mechanism could be responsible for the intracellular changes in these two conditions. Accordingly, ceroid storage disease might be regarded as a disturbance in the metabolism of fat.

SUMMARY

Light microscopic, histochemical and electron microscopic liver changes in two cases of ceroid storage disease in childhood are described. Microscopically, portal cirrhosis and deposition of fat and pigment granules represent the outstanding pathological features. Though pigment granules are most abundant in macrophages in connect... in par...

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Th... ...ance is described

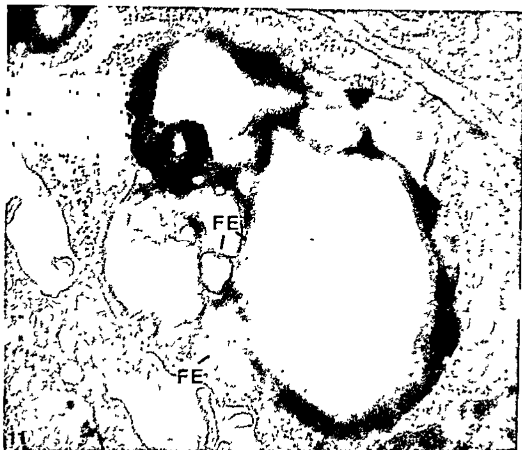


Fig 11
Pigment granule of type III with small osmiophilic ferritin like particles (FE)
× 46 000

chyma were of types I and II, whilst those in granuloma cells, Kupffer cells, and regions transformed into connective tissue were almost exclusively of type III

DISCUSSION

The light microscopic picture of the liver changes found in the cases of the present study are similar to those previously reported in ceroid storage disease in childhood (*Oppenheimer & Andrews* 1959)

The changes on the electron microscopic level— such as fat deposition and dilatation of the endoplasmic reticulum—are interpreted as degenerative. Similar observations have been made in rats with nutritional cirrhosis (*Piccardo & Schwarz* 1958) The presence, as in the first case, of granulomas whose cells contained pigment granules agrees with changes induced in dogs after intravenous administration of fat (*Veglia et al* 1963)

The intracellular pigment granules in both cases exhibit histochemical properties similar to those characterizing lipofuscin granules in the liver at advanced age and the pigment (ceroid) in nutritional deficiency

cirrhosis (cf *Pearse* 1960). It is suggested that the osmiophilic granules on the electron microscopic level represent lipofuscin granules. This inference is based upon comparisons between the occurrence and distribution of the osmiophilic granules in the electron micrographs and lipofuscin granules in the light microscope, its validity is strengthened by the fact that isolated lipofuscin granules show strongly osmiophilic regions in the electron micrographs (*Bjorkerud* 1963). Classified in accordance with Pearse's scale for rating lipofuscin granules by their maturity, the granules observed in granulomas, Kupffer cells and connective tissue regions are more mature than those in parenchymal cells. The electron microscopic classification of osmiophilic granules presented under Results is based on comparisons between their light microscopic location and maturity and the electron microscopic location and appearance. Accordingly pigment granules of types I, II and III on the electron microscopic level represent increasing maturity of the granules, as assessed by light microscopic criteria. Formations resembling pigment granules of types I and II have previously been reported in the liver by various workers including *Essner & Novikoff* (1960) who classified them as lysosomes. Granules of type III have also been described in the liver (*Essner & Novikoff* 1960) and in a variety of other tissues (cf *Bjorkerud* 1963). They are usually known as lipofuscin granules.

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in parenchymal [illegible]
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Their el[illegible] [illegible]ance is described

The liver changes in these two cases of ceroid storage disease are similar to those in experimentally induced pigment deposition Considering the close resemblance of the changes in ceroid storage disease and changes to occur after intravenous fat infusion, it is proposed that ceroid storage disease in childhood may be caused by a disturbance in the lipid metabolism /

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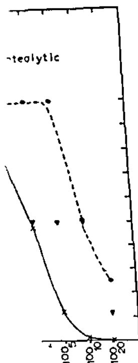
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The present work was underta… …tivity of both crude and purified … determine whether proteolytic e… of importance in this connectio…

MATERIAL

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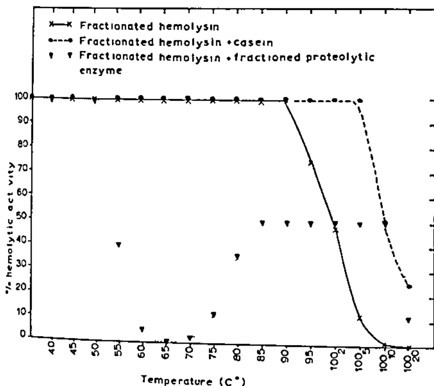


Fig 3

The influence of heat on fractionated haemolysin fractionated haemolysin + casein and fractionated haemolysin + fractionated proteolytic enzyme (produced by *Corynebacterium pyogenes*) pH 6 5 Heat treatment 2 5 10 and 20 minutes at 100° C otherwise 2 minutes

2 minutes at 90° C the haemolytic activity was, in the one experiment, 60 per cent of the original value, in the other about 16 per cent The haemolytic activity remained at this level up to 100° C for 5 minutes By longer exposure to this temperature the activity declined

In Fig 3 are shown the results obtained when DEAE-fractionated haem [illegible]
[illegible] and
pı [illegible] f the
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fractionated haemolysin without any addition started at a temperature slightly above 90° C The saddleshaped course shown by the corresponding experiments with unfractionated haemolysin (Fig 2) did not occur in this case

Addition of protein (sodium caseinate) stabilized the fractionated haemolysin to some extent

Addition of DEAE fractionated bacterial proteinase to the haemolysin gave a similar inactivation curve as that demonstrated for crude

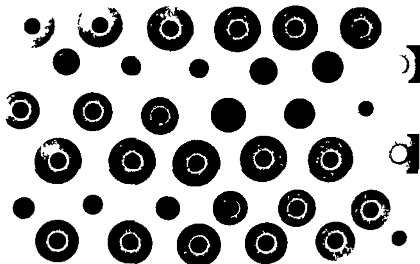


Fig 1

Haemolyzed zones on blood agar plates corresponding to the two heating experiments illustrated in Fig 2 The series of zones continues from the upper left (unheated first and fourth row respectively) to the lower right (100° C for 20 minutes, third and sixth row respectively) The plate is photographed in obliquely transmitted light after 13 hrs incubation at 37° C

—●— Cell free extract from a culture of Bacillus cereus (NVH 1222)
×--× Cell free extract from a culture of Bacillus cereus (NVH 322)

% hemolytic activity

0 10 20 30 40 50 60 70 80 90 100

40 45 50 55 60 65 70 75 80 85 90 95 100_2 100_5 100_{10} 100_{20}

Temperature (C°)

Fig 2

The influence of heat on haemolysin from cell free extracts of two strains of *Bacillus cereus* pH 6 5 Heat treatment 2 5 10 and 20 minutes at 100° C otherwise 2 minutes

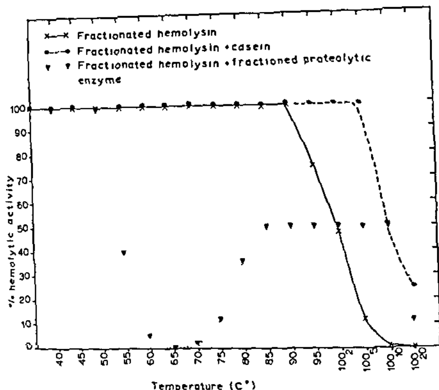


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haemolytic extract (Fig 2) Here it must be emphasized that the thermostability of the mixture is more pronounced at high temperatures than in the case of fractionated haemolysin alone

The ratio between the strength of proteolytic enzyme and the strength of the haemolysin had significance for the result, but this has not yet been studied in detail Preliminary investigations showed that excess of proteolytic enzyme resulted in inactivation of the entire haemolytic activity when the temperature in the mixture for a moment reached 55°–80° C A heat influence of 90°–100° C for 2 minutes might in such cases lead to a total inactivation of the haemolysin On the other hand, a minimum of proteolytic enzyme might lead only to an insignificant inactivation of haemolysin in the interval 55°–80° C

DISCUSSION

It was early observed that the α-toxin of *Staphylococcus aureus* was inactivated by heating at 65° C, but that, paradoxically, the destruction was much less complete at temperatures between 80° and 100° C (*Landsteiner & von Rauchenbichler* (1909), cit *Fulton* 1943)) Since that time this observation has been supported by many investigators *Kodama & Kojima* (1939) found the same for crude β-toxin of *Staphylococcus aureus* These workers examined both α and β-toxin and demonstrated a saddleshaped course of the heat inactivation curve in both cases

Fulton et al have confirmed these observations and have found that the instability was less marked with the purified than with the crude β-toxin They further observed that the lecithinase of *Clostridium perfringens* culture filtrates showed a similar instability at 65° C, and suggest that the phenomenon has general importance (cit *Fulton* 1943)

The saddleshaped heat inactivation curve has recently been demonstrated by *Sandvik* (1962) for crude and DEAE-fractionated proteolytic enzymes

The present work shows that the haemolysin of *Bacillus cereus* also exhibits this character under certain circumstances, that is, when present in a mixture with a proteinase, but not in a purified state High temperatures (100° C for some minutes) led to complete inactivation in all cases This is supposed to be due to a heat denaturation of the enzyme molecules The inactivation at low temperature (55°–80° C) seems to be due to the proteolytic enzyme present, which at this temperature acts by more or less momentary digestion of the haemolysin This is supported by the fact that this effect did not appear when using pure haemolysin *containing no proteinases*

The fact that in the illustrated experiment the haemolytic activity in the mixture with fractionated proteinase is more thermostable at high temperatures than the fractionated haemolysin alone (Fig 3), may be due to impurities in the solution of proteinases added Thus in the

present case the bacterial proteinase originated from a strain of *Corynebacterium pyogenes* As this proteinase was very unstable in a purified state a trace of sodium caseinate was added in order to stabilize it The difference could not be demonstrated in experiments in which proteinase without the addition of caseinate was used These proteinases comprised enzymes produced from fractionated *Bacillus cereus*, unfractionated *Pseudomonas fluorescens* and *trypsin*

SUMMARY AND CONCLUSIONS

1 A crude cell free extract of *Bacillus cereus* haemolysin containing proteolytic enzymes exhibited a saddleshaped inactivation curve of the haemolysin with loss of the haemolytic activity at temperature ranging from 55° 80° C At 90°–100° C the haemolytic activity remained intact to a considerable extent

2 Purified *Bacillus cereus* haemolysin free from bacterial proteinases did not exhibit this character

3 Addition of purified proteolytic enzymes to purified haemolysin caused a heat inactivation curve for the haemolysin corresponding to that obtained with crude proteinase containing haemolysin

4 Addition of sodium caseinate to the purified haemolysin had a certain stabilizing effect on the thermostability

5 The influence of the proteinase on the inactivation curve for haemolysin is discussed

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FROM THE INFLUENZA DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN DENMARK

THE INFLUENCE OF HYDROCORTISONE ON PRODUCTION OF INFLUENZAVIRUS AND INTERFERON IN OVO

By

V. REINICKE

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In 1951 *Kilbourne & Horsfall* showed that chick embryos infected with influenza A or B yielded a higher concentration of virus in the allantoic fluid when the embryos had been inoculated with cortisone (10). In 1953 *Kilbourne & Tateno* employing influenza B virus and cultures of chorio allantoic membranes, observed a similar phenomenon *in vitro* (11). Kilbourne has later investigated the influence of cortisone and other steroids on the mechanism of influenza virus infection in chick embryos (12–17) and in 1961 *Kilbourne et al.* (18) reported that cortisone treatment of chick embryos infected with influenza A PR8 resulted in a decreased production of interferon and also that cortisone treated chorio-allantoic membranes had a lower *in vitro* sensitivity to interferon. Studies of the influence of cortisone on production of influenza viruses and interferon in chick embryos has also been reported by *v. Wasielewski et al.* (23). The present communication is concerned with the influence of hydrocortisone on the production of interferon and influenza B Lee virus in chick embryos.

MATERIALS AND METHODS

Virus. Stock suspensions of influenza virus strains B Lee and A Melbourne were prepared by allantoic inoculation of 11 day old chick embryos. After 48 hours of incubation at 36° C the embryos were cooled and the allantoic fluids were harvested and pooled. The stock viruses were stored at —60° C.

Eggs. Nine to twelve day old embryonated white Leghorn eggs were employed. The eggs were all inoculated by the allantoic route. Since it has been reported (6) that 50 per cent of the inoculated virus will be adsorbed by the cells within 72 minutes it was decided to inject the hydrocortisone into the eggs about 60 minutes after the inoculation of virus (15), in order to avoid an influence on the early phases of the infection.

Haemagglutination (HA) and infectivity titrations. Following the harvest of the

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eggs the allantoic fluids were pooled groupwise and titrated for haemagglutinins according to the method of *Salk* (20). HA-titres to be compared were all measured ... ssed as the average value of three experiments the variation on the repeat titrations a sample of each ... e, to avoid precipitation of urates and stored at +4° C.

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50 γ/ml respectively.

Hydrocortisone. A highly water soluble hydrocortisone compound (di-(17 hydroxy corticosterone 21) phosphoricacidester sodiumsalt (Actocortin, produced by Frederiksberg Chemical Laboratories Ltd. Copenhagen, Denmark) was used in varying concentrations dissolved in distilled water.

Dialysis. Dialysis bags
Carbide International Cor
dialysis was performed a
dialysis took place at +
The surrounding volume
period of dialysis was 48
performed against phosphate buffer.

Shaking machine. A machine working with a stroke of about 10 cm and a frequency of about 70 strokes per minute was employed.

Inactivation. Before the content of interferon of an allantoic fluid containing both influenza virus and interferon can be measured it is necessary to destroy the interfering capacity of the virus. In the present experiments inactivation of the virus was carried out as reported by *Wagner* (22) by heating the allantoic fluids to 65° C for one hour. This procedure destroys the infectivity, the haemagglutinins, and interfering capacity of the virus without reducing the activity of the interferon (22, 4).

Measurement of interferon. The content of interfe...
measured as follows: 10 mm × 20
cut with a pair of scissors from 11

(about
... of inter-
...ed and the membranes were inoculated with challenge virus. This consisted of 1 ml per mem...
This dilution of viru
infectivity titre of 10
tubes were again sto
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... interferon was accordingly expressed as the difference between the logarithms of the average control HA titre and the HA-titre obtained after pretreatment of the membranes with the 1 : 4 dilution of inactivated allantoic fluid.

Some allantoic fluids diluted 1 : 4 were found to be without inhibiting effect on the production of haemagglutinin. In such cases the titres obtained with undiluted fluids were used for calculation (figures shown in brackets in Tables 1, 2 and 4). If the undiluted allantoic fluid was found to be without inhibiting capacity the fluid was considered to contain no interferon.

To obtain an estimate of the accuracy of the method used in measuring interferon an experiment totaling 35 repeated titrations of a 1 : 4 diluted batch of interferon with 12 controls was performed. A standard deviation of 0.15 was found on interferon titres calculated from a single measurement of the batch of interferon and a control value taken as an average of 4 control measurements. Thus a difference in titres exceeding 0.60 can be considered significant at the 95 per cent level.

EXPERIMENTAL RESULTS

In the first series of experiments an optimal effect of hydrocortisone was ensured by injecting the chick embryos every 24 hours with hydrocortisone.

TABLE 1

Virus and Interferon Titres in Allantoic Fluids from Eggs Inoculated with $10^{3.7}$ ID_{50} of Influenza B Lee Virus Prior to Inoculation of Varying Doses of Hydrocortisone

Amount of hydrocortisone per dose	Titres	Hours after inoculation of virus			
		16 hrs	40 hrs	64 hrs	88 hrs
2.5 γ	HA ID_{50} Interferon	<0.30 3.4 0.79	2.28 8.3 1.01	3.48 9.1 1.24	3.26 8.7 1.62
25 γ	HA ID_{50} Interferon	<0.30 3.4 0.34 (0.09)	2.51 8.5 0.04 (—0.04)	3.63* 9.3 0.71	3.33 8.9 1.24
250 γ	HA ID_{50} Interferon	<0.30 3.7 0.19 (0.09)	2.28 8.2 —0.57 (—0.34)	3.48 9.0 —0.04 (—0.11)	3.48 9.0 0.04 (0.04)
None Control	HA ID_{50} Interferon	<0.30 4.2 0.94	2.96 8.6 >2.07	3.18 8.6 >2.07	3.18 8.0 >2.07

All titres are recorded as $\log_{10}$
* See text
Figures in brackets: see text

A total of 160 9–12 day old chick embryos was used in each experiment: *A* 40 chick embryos 9 days old, *B* 40 chick embryos 10 days old, *C* 40 chick embryos 11 days old and *D* 40 chick embryos 12 days old. All eggs were inoculated with 10^{3} ID_{50} of influenza *B Lee (0.2 ml)* followed after one hour's incubation at 37° C by inoculation of 0.5 ml of hormone suspension. The eggs were all harvested when the embryos were 13 days old, *i.e.* group D was harvested after 16 hours of incubation, group C after 40 hours, group B after 64 hours and group A after 88 hours of incubation. After inoculation of virus the eggs were treated groupwise

in the following manner. 10 eggs were inoculated with physiological saline (control). The remaining 30 eggs were divided into groups of 10 eggs each and inoculated with 0.5 ml amounts of hydrocortisone containing 2.5 γ, 25 γ and 250 γ of hormone, respectively. The inoculation of hydrocortisone (or control fluid) was repeated every 24 hours until the harvest of the eggs took place. The allantoic fluids were pooled groupwise and immediately titrated for haemagglutinin and infectivity. A sample of each pooled allantoic fluid was dialyzed 48 hours immediately after the harvest, in order to remove the hydrocortisone. After dialysis the fluids were heat inactivated and the interferon content of the 12 pools was measured simultaneously. The results of a representative experiment appear in Table 1.

It can be seen that the steroid treated eggs contain less haemagglutinin in the early phases of infection, while the final HA-titres are somewhat higher (significant difference > 0.15 $\log_{10}$) than the control titres (Table 1 *). It should be noticed, however, that the control titres in this experiment are somewhat low. Usually in this laboratory, HA-titres around $10^{3.48}$ are obtained for influenza B Lee after 48 hours of incubation.

The infectivity titrations of allantoic fluids from the steroid treated eggs and the control eggs did not reveal any significant differences.

It can be seen that hydrocortisone, in the doses used in this experiment, markedly decreased the content of interferon in the allantoic fluids. The effect was observed in eggs inoculated with 2.5 γ hydrocortisone and was more pronounced with increasing dose. In eggs treated with 25 γ hydrocortisone per dose, no interferon could be demonstrated in the allantoic fluid until 40 hours after inoculation of virus, and in eggs which received 250 γ hydrocortisone per dose no interferon could be detected during the entire experimental period.

To investigate the influence of a single injection of hydrocortisone in contrast to repeated injections, the following experiment was performed. Three groups A, B and C, each consisting of 40 ten-day-old chick embryos were employed. The eggs were all inoculated with $10^{3.3}$ ID_{50} of influenza B Lee. After one hour's incubation at 37° C the eggs of each group were divided into 4 subgroups and treated as follows: 10 eggs (control) were inoculated with physiological saline, 10 eggs received 2.5 γ hydrocortisone, 10 eggs 25 γ hydrocortisone and 10 eggs 250 γ hydrocortisone. Hereafter the eggs were again incubated at 37° C. The eggs in group A were harvested after 64 hours and the allantoic fluids from each subgroup of 10 eggs were pooled. 24 hours after the first injection of hormone the group B eggs received one more dose of hormone (controls received saline) followed by reincubation at 37° C. Harvest and pooling of the allantoic fluids from the subgroups of the B eggs took place after 64 hours. The group C eggs received one more dose of hormone 24 hours after the first dose and another dose 48 hours after the first. Harvest and pooling of the C eggs took also place after

64 hours of incubation The infectivity titres obtained in the control eggs hardly represents the maximum titres obtained in the control eggs since the eggs all have been harvested after 64 hours incubation With an inoculum of the size used in this experiment the maximum infectivity titre is usually obtained after 44 hours incubation, after which the titre remains high for some hours and then decreases In order to determine the maximum titres in the control eggs, a second control experiment using two groups each consisting of ten 10-day-old chick embryos, was run parallel with the above described experiment These eggs received $10^{3.3}$ ID_{50} of influenza B Lee virus followed of control fluid once (after one hour's incubation) or twice (after one hour's incubation and 24 hours after the first injection) Harvest and groupwise pooling of the allantoic fluids took place after 44 hours

All pools of allantoic fluids were titrated for haemagglutinin and infectivity The 12 pools from eggs harvested after 64 hours incubation were dialyzed for 72 hours, after which the content of interferon was measured The results are shown in Table 2

TABLF 2

Virus and Interferon Titres in Allantoic Fluids from Eggs inoculated with $10^{3.3}$ ID_{50} of Influenza B Lee Virus Prior to Inoculation of Varying Doses of Hydrocortisone

Amount of hydro-cortione per dose	Titres	Number of doses applied during period (64 hrs) of incubation		
		1 dose	2 doses	3 doses
2 5 γ	HA	3 48	3 48	3 56*
	ID_{50}	9 1	8 9	9 3
	Interferon	1 33	0 81	0 73
25 γ	HA	3 56*	3 33	3 33
	ID_{50}	9 3	8 9	8 7
	Interferon	1 26	0 58 (0 96)	0 21 (0 36)
250 γ	HA	3 18	3 11	3 07
	ID_{50}	9 5	8 3	8 7
	Interferon	0 88	—0 08 (0 36)	0 03 (0 03)
None Control	HA	3 33 3 26§	3 11 3 26§	3 07
	ID_{50}	8 9 9 0§	8 5 8 9§	8 5
	Interferon	2 09	1 71	1 71

All titres are recorded as $\log_{10}$
* See text
Figures in brackets see text
§ 44 hours period

In accordance with the first experiment (Table 1) a tendency towards a higher final HA-titre in eggs treated with hydrocortisone (Table 2) can be seen This is the case whether the eggs have received one, two or three doses during the experimental period Furthermore it can be

seen that there is no systematic variation between the titres from eggs which have received one, two or three doses.

It can also be seen that a single dose of hydrocortisone applied shortly after the inoculation of virus, reduces the content of interferon in the allantoic fluid during the entire period of incubation. Furthermore the results obtained with doses of 25 γ and 250 γ show that repeated injections further reduce the content of interferon. This is most marked with the 250 γ dose where no interferon could be detected after two injections. With the 2.5 γ dose hardly any difference could be seen as regards the content of interferon in the allantoic fluids from eggs treated with one, two or three doses respectively.

It should be considered whether the hydrocortisone effect on the content of interferon of allantoic fluids, as observed in the two experiments described above, is actually due to a diminished content of interferon or whether it might be due to a hydrocortisone induced reduction of the activity of the interferon present in the allantoic fluid.

To investigate this possibility the following experiment was performed. Three batches of interferon consisting of inactivated allantoic fluid were employed. From each batch of interferon 25 ml were distributed into each of two Brockway bottles. One of the two Brockway bottles received 0.5 mg of hydrocortisone giving a final concentration in the allantoic fluid of 20 γ per ml. This concentration has been calculated as the maximum concentration of hydrocortisone obtained in the allantoic fluid in the present study. Assuming that the hydrocortisone injected into an egg is evenly distributed throughout the egg and assuming that no injected hydrocortisone is metabolized, the theoretical maximum content of hydrocortisone in an egg must be 4 × 0.25 mg corresponding to 4 injections of 250 γ hydrocortisone. If the volume of an egg is estimated to be 50 ml, the theoretical maximum concentration of hydrocortisone is calculated to 20 γ per ml allantoic fluid.

TABLE 3

The Stability of Interferon during Incubation with Hydrocortisone

Incubation hours	Before treatment	Incubation with hydrocortisone followed by dialysis	Incubation with saline followed by dialysis
24	1.86	1.33	1.49
48	1.18	1.03	0.95
72	1.18	1.03	1.03

All titres are recorded as $\log_{10}$

The other Brockway bottle serving as control received 1 ml of physiological saline. The Brockway bottles were placed in a shaking machine at 36° C and were removed two at a time from the machine after 24, 48 and 72 hours of incubation. Immediately after removal from the shaking machine the fluids were dialyzed for 72 hours. Following

dialysis the content of interferon was measured in the dialysates as well as in the non-dialyzed fluids The results appear in Table 3

It can be seen that the titres of interferon after incubation and dialysis are not different from the titres of the untreated fluids, indicating that hydrocortisone has no influence upon the activity of interferon

Thus the low content of interferon in allantoic fluids, observed in the present experiments (Tables 1 and 2) can not be ascribed to a hydrocortisone induced destruction of interferon

Having investigated the influence of hydrocortisone upon the inhibiting effect of preformed interferon, it seemed of interest also to investigate the influence of hydrocortisone upon the test system for interferon The following experiment was performed 288 chorio allantoic membrane pieces were distributed into 12 Brockway bottles with 24 membrane pieces per bottle To each bottle was added 24 ml of Earles BSS containing one of the following concentraions of hydrocortisone 0 γ/ml, 0 5 γ/ml, 1 γ/ml, 5 γ/ml, 50 γ/ml and 250 γ/ml, 2 bottles were used for each concentration of hydrocortisone

The bottles were placed in a shaking machine and incubated for 24 hours at 37° C The membranes were then washed in 5 × 100 ml Earles BSS per 24 membranes and, in the usual manner, employed for measuring the interferon in a batch of inactivated allantoic fluid For each concentration of hydrocortisone, 24 membranes were incubated with dilutions of allantoic fluids while the other 24 membranes were inbubated with Earles BSS and served as controls

TABLE 4

The Influence of 24 Hours of Incubation with Hydrocortisone upon the Chorio Allantoic Membrane Sensitivity to Interferon

Concentration (γ ml) of hydrocortisone	Titre of interferon
0 (control)	1 50
0 5	1 00
1	0 81
5	0 57 (1 10)
50	0 68
250	0 49 (0 79)
No incubation	1 20

All titres are recorded as $\log_{10}$
Figures in brackets see text

In the same experiment the titre of the interferon was also measured by using fresh chorio-allantoic membrane pieces The results are recorded in Table 4, from which it can be seen that the titre of the interferon bach was found to be lower when membranes treated with hydro cortisone were used instead of fresh membranes In other words, addition of hydrocortisone to the allantoic cells decreased the sensitivity of these cells to interferon In the present experiment the effect became

marked with membranes incubated with hydrocortisone in concentrations between 0.5 γ/ml and 1 γ/ml. A complete desensitization of the membranes to interferon was not observed. Even membranes incubated with hydrocortisone in a concentration of 250 γ/ml showed a certain sensitivity to interferon. It can also be seen that the sensitivity to interferon was identical for membranes incubated with the following concentrations of hydrocortisone: 5 γ/ml, 50 γ/ml and 250 γ/ml.

In the allantoic fluids from hydrocortisone treated eggs a certain amount of hydrocortisone is most likely present. The possible influence of this compound upon the assay methods was investigated in the following way.

TABLE 5

A Series of 5 HA Titrations of a 1:10 Dilution of Influenza B Lee Stock Virus Performed in the Presence of Varying Concentrations of Hydrocortisone in the Erythrocyte Suspension

Concentration (γ ml) of hydrocortisone	Experiment number				
	1	2	3	4	5
0	3.03	3.03	3.03	3.03	2.96
20	3.03	3.03	3.03	2.96	2.96
40	2.96	3.03	2.96	3.03	3.03
60	3.03	3.03	3.03	3.03	3.03

All titres are recorded as $\log_{10}$.

TABLE 6

The Influence of Hydrocortisone upon the Titres Obtained in Infectivity Titrations of Four Different Stocks of Influenza B Lee Virus

HA Titre of virus stocks	Concentration (γ ml) of hydrocortisone	ID_{50}
3.03	20	9.8
	none	9.0
3.26	20	9.0
	none	9.0
3.26	20	8.8
	none	8.6
3.26	100	8.7
	none	8.8

All titres are recorded as $\log_{10}$

An influenza B Lee stock diluted 1:10 was repeatedly HA titrated with and without hydrocortisone in the diluent. For convenience hydrocortisone was added to the suspension of erythrocytes immediately before this suspension was distributed into the test tubes. The results are shown in Table 5. The theoretical maximum concentration of hydrocortisone in the allantoic fluid 20 γ/ml as well as 2 and 3 times this concentration were found to have no influence on the titres obtained in HA titrations.

A hydrocortisone concentration of 20 γ/ml is also without influence upon the titres obtained in infectivity titrations as appears in Table 6, which shows infectivity titrations of different virus stocks with and without hydrocortisone A single experiment was performed with 100 γ/hydrocortisone/ml, a concentration which also proved to be without influence on the infectivity titre In these last-mentioned experiments the hydrocortisone was dissolved in the phosphate-broth employed for serial dilution of virus in infectivity titrations, thus the desired concentration of hydrocortisone was ensured throughout the dilution series

DISCUSSION

The *present experiments have shown that hydrocortisone has a* marked influence on the content of interferon in the allantoic fluid from eggs inoculated with influenza B Lee virus This observation is in disagreement with results otained in a similar study by *v Wasielewski et al* (23) No explanation of this discrepancy can be offered on the basis of the available data However the present findings agree well with those reported by *Kilbourne et al* (18) who also found a decreased yield of interferon in cortisone treated eggs Whether the decrease or even absence of interferon observed in the present study is due to a decreased cellular synthesis of interferon or it is due to a hormone induced change in the permeability of the allantoic cell membranes tending to withhold the interferon in the cells has not been fully established However, *Kilbourne et al* (18) have reported that chorio-allantoic membranes from virus inoculated eggs treated with cortisone have a lower content of interferon This observation together with the findings described in the present study seems to indicate a decreased synthesis of interferon induced by cortisone

In accordance with Kilbourne's results (15 17) a hydrocortisone-induced decrease of the HA-titres was seen until 40 hours after the virus inoculation *in chick embryos infected with influenza B Lee virus* The higher final influenza HA-titer also described by *Kilbourne* (10, 12, 15 16) and by *v Wasielewski et al* (23) was on the other hand not very *marked in* the present experiments—although a tendency towards a somewhat higher titre was seen (Tables 1 and 2 *) It seemed possible that this discrepancy might be due to the repeated injections of hydrocortisone, since *Kilbourne* and *v Wasielewski et al* employed only a single injection of steroid (10, 12, 15, 16, 17, 23) However, the effect was observed whether the eggs had received one or several injections of hydrocortisone (Table 2)

Another explanation of the discrepancy could be a toxic cell damaging effect of the hydrocortisone compound used in the present experiments (17) However, the fact that the HA-itres in eggs which had received 3-4 doses of 250 γ hydrocortisone (*Tables 1 and 2*) were *identical* with those of the controls, or even slightly higher, excludes this possibility

Earlier experiments by *Fong & Louie* (3) and *Kilbourne* (13) employing single doses of hydrocortisone acetate as large as 7.5 mg per egg and 1 mg per egg respectively, are also compatible with this conclusion.

The experiments in which 3 doses of 2.5 γ hydrocortisone were employed (Table 2) shows that it was possible, by repeated injections of a total amount of 7.5 γ hydrocortisone, to obtain an allantoic fluid with a slightly increased HA-titre but also with significantly less interferon. This observation seems to indicate selective influence by hydrocortisone upon the protein synthesizing mechanisms of the chick embryo rather than a toxic effect. This concept is also in accordance with a report from 1951 by *Stock et al.* (21) that 15 γ hydrocortisone acetate per egg is the minimum dose required for inhibition of the growth of the chick embryo.

Kilbourne (16) suggests that the effect of hydrocortisone on the synthesis of influenza virus is an inhibition which nevertheless results in a higher final production of influenza virus because hydrocortisone also inhibits the autointerference of virus in the later phases of the infection. The discrepancy between Kilbourne's observations and those obtained in the present study might possibly be explained by the hydrocortisone compound di(17-hydroxycorticosterone-21-)phosphoriacid-ester sodiumsalt used in the experiments described above. If this compound has a particularly marked inhibiting effect on the synthesis of haemagglutinin, or a less marked inhibiting effect on the autointerference of the virus, compared to other previously used cortisone and hydrocortisone compounds (10, 12, 15, 16, 17, 23) the final result would not necessarily have to be an increased production of haemagglutinin.

Concerning the infectivity titration of allantoic fluids from eggs treated with hydrocortisone (Tables 1 and 2) no systematic variations from controls could be shown. This observation is in disagreement with observations by *Kilbourne & Horsfall* (10) who found higher infectivity titres as well as higher HA-titres of influenza virus in eggs treated with 11-dehydro-17-hydrocorticosterone-21-acetate.

It is interesting that the catabolic steroid hydrocortisone seems to inhibit the synthesis in ovo of interferon as well as of influenza virus in the early phases of infection. Since virus consists mainly of proteins and interferon is also a protein, the inhibition of the synthesis of these compounds is in accordance with the general decrease of proteinsynthesis induced by hydrocortisone *in vivo* (8, 9).

The experiments recorded in Tables 1 and 2 showing that an influenza HA titre of about 10^3 could be obtained in allantoic fluid in which no interferon could be detected seems to indicate a marked selective inhibition of the synthesis of the protein interferon together with a relatively less marked inhibition of proteins entering influenza virus particles.

It might also be considered whether the effect of hydrocortisone on the production of interferon could be secondary to the effect on the

virus synthesis, in that the hydrocortisone induced a slower production of virus in the early phases of infection and that this resulted in a decreased production of interferon However, doses of hydrocortisone varying from 2 5 γ to 250 γ were found to delay the appearance of virus in the allantoid fluid to the same extent while the concentration of interferon decreased with increasing doses of hydrocortisone (Table 1)

The present observations on hydrocortisone induced inhibition of interferon are consistent with other observations on the effect of steroids on virus infections For instance, it is wellknown that cortisone treated animals are less resistant to virus infections (9) a phenomenon which may well be due to a steroid induced inhibition of interferon production in the animals (7)

A more violent course of infection following addition of hydrocortisone has also been observed in virus infected tissue cultures *Holden & Adams* (5) found in vaccinia virus infection of L-cells treated with hydrocortisone, a faster and more pronounced cythopathogenic effect, earlier appearance of intracellular viral antigen as demonstrated by immunofluorescence technique and a higher titre of virus, observations which all are compatible with the theory of a hydrocortisone induced inhibition of synthesis of interferon

The observation of a hydrocortisone induced decreased cellular sensitivity to preformed interferon (Table 4) is in accordance with the report from 1961 by *Kilbourne et al* (18) Decreased sensitivity to interferon could also serve as an explanation of the more violent course of infection in the presence of hydrocortisone The hydrocortisone has hardly any toxic effect on the membrane pieces With increasing concentrations of steroid the membranes showed decreased sensitivity to interferon and simultaneously a tendency to increased production of haemagglutinin following addition of challenge virus, indicating that the synthesizing mechanisms of the allantoic cells were, at least partially, intact in the hydrocortisone treated membranes

SUMMARY

Administration of hydrocortisone to chick embryos inoculated with $10^{3\ 3}$ ID_{50} $10^{3\ 7}$ ID_{50} of influenza B Lee virus resulted in a lower content of interferon in the allantoic fluids This phenomenon which most likely was due to a reduced synthesis of interferon was observed when a dose of 2 5 γ hydrocortisone was employed The effect was enhanced by larger doses and by repeated injections of hydrocortisone, and became most marked with 3 4 doses of 250 γ hydrocortisone when no interferon could be detected in the allantoic fluids during the experimental period

Only a slight tendency towards an increased virus titre was observed in the allantoic fluids from eggs treated with hydrocortisone

A decreased sensitivity to interferon was demonstrated in *in vitro*

experiments with chorio-allantoic membrane pieces incubated for 24 hours with hydrocortisone

The relationship between the hydrocortisone effect on production of influenza virus and interferon in ovo is discussed

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FROM THE UNIVERSITY INSTITUTE OF FORENSIC MEDICINE, COPENHAGEN
(HEAD: PROFESSOR H. GORMSEN, M.D.) DEPARTMENT OF SEROLOGY,
(HEAD: K. HENNINGSEN, M.D.)

ON THE TRANSFORMATION OF GC INTO AN ALPHA-1 GLOBULIN BY DISINTEGRATED BLOOD CELLS AND PROTEOLYTIC ENZYMES

By

B. Nerstrom

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In immunoelectrophoretic analysis of dried blood stains performed at our laboratory we observed even in rather fresh stains a weakening of the Gc components and the appearance of a new precipitate, immunologically identical with Gc, within the alpha-1 globulin region (5). The electrophoretic position of this new precipitate was identical in blood stains from donors belonging to each of the three Gc groups. In dried serum stains no change occurred within the first 4 weeks. Similar changes of the Gc components were observed in a few serum samples sent from Greenland.

Since then the same changes have been observed in several cases on examination of badly stored serum. Most samples were haemolyzed but it should be mentioned that haemolysis generally does not affect Gc grouping. As dried blood stains and haemolyzed blood samples seem to have a disintegration of the blood cells in common, the hypothesis was advanced that the changes of the Gc components are induced by one or more enzymelike substances released from disintegrated blood cells. This hypothesis was tested by examination of a series of blood samples subjected to freezing-thawing procedures and of serum incubated with frozen erythrocytes, leucocytes and thrombocytes. In addition the effect of disintegrated yeast cells and of a series of pure enzyme preparations was examined. A brief report (3) on the results was given recently. This paper gives the results in detail.

MATERIAL AND METHODS

Reagents

Whole blood, serum and isolated blood cells were obtained from 3 healthy adult females of group Gc 1-1, 2-1 and 2-2 respectively. In a few exceptional cases experiments were performed on blood from members of the staff and some pure leuco-

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cyte preparations originated from donors at the Blood Bank, University Hospital, Copenhagen

Serum was separated from the clot after storage at 4° C overnight and the serum samples were kept at —20° C until they were used

Erythrocytes were isolated from citrate blood The cells were suspended in saline and centrifuged at 3000 r p m for 10 minutes 5 to 7 times The upper cell layer containing leucocytes and thrombocytes was carefully removed after each centrifugation and it was checked by microscopy that the final preparations contained red cells only

integration of the cells leucocytes were isolated from defibrinated blood too

Thrombocytes were isolated from plasma using test tubes pipettes etc of perspex or using utensils covered with paraffine The cells were suspended in saline with citrate added and centrifuged at 4000 r p m for 20 minutes 3 times The washed cells from each of the three donors were suspended in saline The suspensions contained 5-600 000 5-600 000 and 2-300 000 thrombocytes respectively, and less than 10 erythrocytes and leucocytes per microliter[2]

Yeast cells were common baker's yeast from *De Danske Spritfabrikker*

Enzymes used were commercial preparations most of them kindly furnished by *Novo s Terapeutical Laboratory*

Procedures

Whole blood and *citrate blood* from the donors of type Gc 1 1, 2 1 and 2-2 was *frozen* and thawn 2 to 3 times These samples and non-frozen controls were kept at 37° C and after 2 and 4 hours serum and plasma samples were withdrawn for immunoelectrophoretic analysis In an additional experiment on whole blood from the same three donors the samples were kept at 4 22 and 37° C and analysis was performed after 3 and 23 hours

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Thrombocytes 200 microliters of Gc 1-1 serum were incubated with 200 microliters of each of the three cell suspensions containing 5 600 000 5 600,000 and 2 300 000 cells per microliter, *respectively* 200 microliters of serum of type Gc 1 1 2 1 and 2 2 were incubated with 200 microliters of a suspension of pooled cells containing about 450 000 cells per microliter

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[1] kindly performed by *Gert Jensen* M D the Blood Bank University Hospital Copenhagen

[2] Preparation and counting ...
M D the Central Laborato...

[3] kindly performed by ...

FROM THE UNIVERSITY INSTITUTE OF FORENSIC MEDICINE, COPENHAGEN
(HEAD: PROFESSOR H. GORMSEN, M.D.) DEPARTMENT OF SEROLOGY
(HEAD: K. HENNINGSEN, M.D.)

ON THE TRANSFORMATION OF GC INTO AN ALPHA-1 GLOBULIN BY DISINTEGRATED BLOOD CELLS AND PROTEOLYTIC ENZYMES

By

B. NERSTRØM

Received 30. viii. 63

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integration of the cells leucocytes were isolated from defibrinated blood too

Thrombocytes were isolated from plasma using test tubes pipettes, etc of perspex or using utensils covered with paraffine The cells were suspended in saline with citrate added and centrifuged at 4000 r p m for 20 minutes 3 times The washed cells from ... ontained ... 10 cry

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from each of the three donors were suspended in saline The suspensions contained 5–600 000 5–600 000 and 2 300 000 thrombocytes respectively and less than 10 erythrocytes and leucocytes per microliter[2]

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[2] Preparation and counting of thrombocytes was kindly performed by *J C Amris* M D the Central Laboratory University Hospital Copenhagen

[3] Kindly performed by I *Hartelius* Ph D The Carlsberg Laboratory Copenhagen

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The non frozen controls did not display any changes of the Gc precipitates within the experimental period

Serum Incubated with Frozen Erythrocytes

Serum incubated with disintegrated packed red cells in up to equal volumes displayed no clear-cut changes of the Gc precipitates, even after storage at 37° C for 18 hours A slight anodic drawing out of the Gc 2–1 precipitates was observed occasionally, but no definite weakening of the Gc components was present

Serum Incubated with Frozen Leucocytes

In a preliminary experiment it was demonstrated that disintegrated leucocytes were able to provoke changes of the Gc components similar to those observed in frozen whole blood

Further experiments showed that the whole range of transformations *from a slight anodic extension of the normal Gc precipitate to a complete* disappearance of the latter and the formation of a pronounced new alpha-1 precipitate occurred in serum incubated with equal volumes of disintegrated leucocytes in varying dilutions Thus disintegrated packed leucocytes diluted 1 100 with saline provoked a weakening of the Gc components, most markedly of the 2-component, after 3 hours' incubation at 37° C With a dilution 1 50 both components had completely disappeared from serum and were substituted by an intense new alpha-1 globulin component

The immunological identity of the new alpha-1 globulin in serum from each of the three donors was verified by admixture of fresh Gc 1 1 serum prior to the electrophoretic separation Furthermore, it was demonstrated that such samples, showing no normal Gc precipitate but an intense new alpha-1 precipitate, were found able to remove anti Gc from immune sera after incubation of equal volumes at room temperature for 1½ hours

Supplementary investigations with leucocytes isolated from defibrinated blood gave similar results The alteration of the Gc components were recognizable in serum incubated for 3 hours with equal volumes of a frozen cell suspension containing about 7,000 leucocytes per microliter

The ability of the disintegrated leucocytes to transform the Gc components into a new alpha 1 globulin was found to remain in the supernatant after centrifugation The active factor was destroyed by heating in a boiling water bath for 10 minutes

Serum incubated with non-frozen leucocytes in dilution 1 25 in saline at 37° C displayed no changes of the Gc precipitates even after 17 hours The immunoelectrophoretic pattern of disintegrated leucocytes showed only a single fuzzy precipitate at the site of albumen

Immunoelectrophoresis of the serum samples was performed as described in detail elsewhere (4) The duration of the electrophoretic separation was 2 hours and the voltage gradient 6 to 7 volts per cm Diffusion took place at 37° C for 16 to 24 hours against polyvalent rabbit anti-human sera containing antibodies against the Gc proteines (R 146, R 214 and R 228) In some cases an anti-Gc serum prepared in horse (Behring Werke no 3438 P) was used

Preliminary *reading of the results* was carried out on the native preparations Further analysis of the patterns however, was based upon the amido black stained preparations and the film negatives of the native preparations

RESULTS

Frozen Whole Blood

The immunoelectrophoretic patterns of the blood samples subjected to freezing-thawing prior to the storage deviated from the normal serum patterns in the following respects The migration of the haptoglobins was reduced, as it is seen in sera with haemoglobin added, and an extra precipitate appeared in the beta globulin region when antisera containing anti-haemoglobin were used As far as the Gc are concerned a series of characteristic changes occurred Examples are given in Fig 1 and Fig 2

The distance from the Gc precipitates to the antiserum trough was increased indicating a reduction in concentration of the Gc components in serum The characteristic electrophoretic mobilities of the Gc-1 and Gc-2 components, however, were not affected to any significant degree The concentration of the 2-component was reduced more than the concentration of the 1-component in samples kept under similar conditions In the alpha-1 globulin region a new precipitate appeared showing reaction of immunological identity with the Gc components The electrophoretic position of this particular component was the same in sera of type Gc 1–1, 2 1 and 2–2

These alterations of the patterns were present in frozen blood samples after 2 hours' storage at 37° C Re-examination of the samples after 4 hours showed that especially the concentration of the 2-component decreased during prolonged storage and that the concentration of the new alpha-1 globulin increased

The changes were reproducible on new samples from the same three donors After 23 hours the 2 component had completely disappeared from the patterns and the 1-component was very weak

The results of storing of the samples at 4, 22 and 37° C demonstrated that the transformation of the Gc proteins was accelerated at higher temperatures

The changes of the Gc precipitates including the occurrence of a new alpha-1 precipitate could be demonstrated with any of the rabbit antisera used, whereas the new alpha-1 precipitate was weak or not present when the anti-Gc serum prepared in horse was used

No differences between the patterns of frozen whole blood and frozen citrate blood were observed

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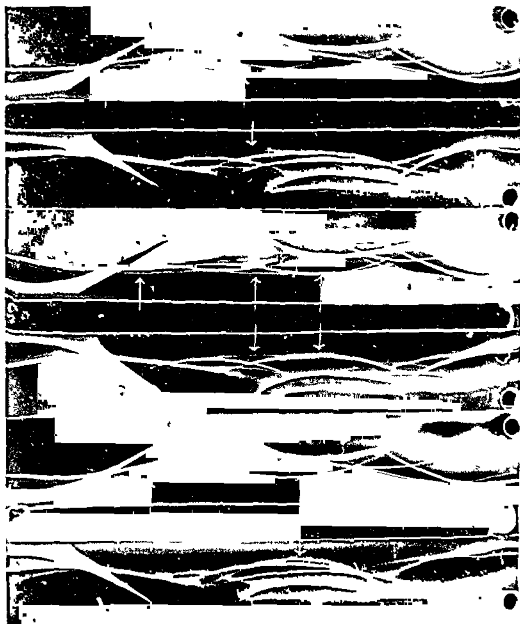


Fig 1

Immunoelectrophoretic patterns displayed by serum from frozen and non frozen blood samples after 3 hours storage at room temperature —*A* Frozen blood of type Gc 1-1 —*B* Non frozen blood of type Gc 1 1 —*C* Frozen blood of type Gc 2 1 —*D* Non frozen blood of type Gc 2 1 —*E* Frozen blood of type Gc 2 2 —*F* Non frozen blood of type Gc 2 2 —Antiserum used throughout a rabbit anti human serum (R 228) The arrows indicate the Gc components and the new alpha 1 globulin

Serum Incubated with Frozen Thrombocytes

As in the case with leucocytes, disintegrated thrombocytes were found to induce the characteristic changes of the Gc precipitates as observed in frozen whole blood

In the first part of the experiment, in which suspensions of disinte-



Fig 2

Immunoelectrophoretic patterns displayed by serum from the same frozen and non frozen blood samples as shown in Fig 1 after 24 hours storage at room temperature

grated thrombocytes from each of the three donors were added to Gc 1–1 serum in equal volumes it was demonstrated that two suspensions with 5 600 000 cells per microliter provoked an intense alpha 1 precipitate and a marked reduction in concentration of the normal Gc component, whereas the cell suspension with 2 300 00 cells per microliter induced anodic extension of an almost normal Gc precipitate, only, after 3 hours'

incubation at 37° C Re-examination of the samples after 18 hours showed only slight accentuation of the alteration

Experiments, in which serum of type Gc 1-1, 2-1, and 2-2 was incubated with pooled disintegrated thrombocytes under similar conditions, it was noted that the concentration of the 2 component was reduced more than the concentration of the 1-component

In control samples intact thrombocytes induced no changes of the Gc components, and the suspensions of disintegrated thrombocytes displayed no precipitates with the antisera used

Finally, it was demonstrated that the active factor in the thrombocytes remained in the supernatant after centrifugation of the frozen and thawn suspensions

Serum Incubated with Yeast Cells and Enzymes

The effect of disintegrated yeast cells and of a series of proteolytic and saccharolytic enzymes was tested As the analysis of each individual preparation was not exhaustive, only the positive results with yeast and a bacterial proteinase isolated from a subtilis strain will be given

Two hundred microliters of serum incubated for 2 hours at 37° C with 2 microliters of disintegrated yeast cells displayed no changes of the Gc precipitates, 20 microliters of yeast cells induced a slight anodic extension of the precipitates, and 200 microliters removed the normal Gc precipitates In the alpha-1 globulin region a precipitate similar to that occurring in frozen blood was demonstrated, but the immunological identity with Gc was not established Intact yeast cells had no effect on Gc

Incubation with bacterial proteinase, 1 mg in 1 ml serum at 37° C for 25 minutes and 0 2 mg in 1 ml serum at 37° C for 1½ hours, gave similar results

The action of yeast cells and bacterial proteinase was not as elective as the action of the blood cells, changes occurring in other components, too

DISCUSSION

The results of the present investigation clearly indicate that freezing of whole blood induces a gradual transformation of the Gc components of serum into a single alpha 1 globulin during subsequent storage, and that the 2-component is more liable to be transformed than the 1-component As Gc in serum are unchanged even after several freezing-thawing procedures and prolonged storage the transformation must be conditional on the presence of blood cells

Incubation of serum with different pure cell preparations demonstrated that the transformation was induced by a factor released from the disintegrated cells The factor was present in leucocytes and thrombocytes, but not or in a very small concentration only in erythrocytes

Inactivation of the factor by heating strongly suggests that the factor is an enzyme. As leucocytes, thrombocytes and yeast cells are known to contain proteolytic enzymes, and as a crystalline proteolytic enzyme gave rise to changes of Gc similar to those provoked by the cells, it is furthermore likely that the active factor is a proteolytic enzyme.

The results agree with those achieved by *Reinskou* (6) in Gc grouping of serum from stored blood and frozen blood. In these experiments frozen blood samples displayed a weakening of Gc, but no new alpha-1 globulin occurred, probably due to a very short storage at room temperature after freezing. The explanation why Gc grouping was possible of blood, severely haemolyzed, during prolonged storage at room temperature (about 4 weeks) may be that the active factor in such samples may be released so slowly that the enzyme concentration owing to continuous inactivation will not become high enough to give rise to demonstrable changes.

The occurrence of a new alpha-1 globulin, immunologically identical with Gc, is observed by *Hirschfeld & Nilsson* (2) in umbilical cord sera of type Gc 2–1 from children with a high degree of bilirubinaemia, mostly due to Rh immunization. An early disintegration of the blood cells in such samples might explain the occurrence of the new alpha-1 globulin but so far this question must be considered unsettled.

The deviating Gc type, designated Gc 2–1 v, described in Chippewa Indians by *Cleve & Bearn* (1) bears much resemblance to the asymmetric Gc 2 1 precipitate induced by disintegrated blood cells. No new alpha-1 precipitate, however, was described in the sera of type Gc 2 1 v but this might depend on the antisera used. It is remarkable that the 2 1 patterns were reproducible on new samples and, furthermore, that three of the Gc 2 1 v variant individuals were members of one family. This seems to indicate a genetical control of the variant, but the genesis of the transformation has not yet been investigated exhaustively.

In connection with these Gc 2–1 variants it must be mentioned that by the use of anti Gc sera prepared in horse a deteriorated blood sample from a donor of type Gc 2–1 may erroneously be grouped as Gc 1–1, owing to the more pronounced reduction in concentration of the 2 component. Such errors, however, can be avoided if potent anti Gc sera giving the new alpha-1 precipitate are used in examinations of samples showing signs of deterioration.

As conclusion, it may be stated that the formerly observed transformation of Gc into an alpha 1 globulin immunologically identical with Gc can be induced by an enzyme-like factor which is pres[illegible]

[illegible] samples, and even for the abnormal patterns observed in serum from non haemolyzed blood samples as such serum may contain a similar factor produced by bacteria. As far as similar [illegible]

deviating patterns of bilirubinemic children and Chippewa Indians are concerned the problem of the genesis must be considered unsettled

SUMMARY

Experiments were performed to test the advanced hypothesis that a characteristic, deviating Gc pattern formerly observed in dried blood stains and certain serum samples is induced by a factor released from disintegrated blood cells

In the present investigation disintegration of the cells was provoked by freezing-thawing procedures

The results demonstrated that the Gc components of serum incubated with disintegrated leucocytes and thrombocytes undergo a gradual transformation into an alpha-1 globulin immunologically identical with Gc, and that the Gc 2-component is more liable to be transformed than the Gc 1-component Furthermore, as similar changes were induced by disintegrated yeast cells and a bacterial proteinase isolated from a subtilis strain, the results indicated that the active factor probably is a proteolytic enzyme

The results are discussed with special reference to the possible existence of similar Gc patterns developed on a genetical basis

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FROM THE DEPARTMENT OF ANTIBIOTICS STATENS SERUMINSTITUT, COPENHAGEN

THE IN VITRO ACTIVITY OF FRAMYCETIN

Spectrum, Sensitivity Test and Cross Resistance

By

JES FORCHHAMMER

Received 24 ix 63

The antibiotic framycetin,[1] available as monosulphate Soframycin®, is produced by some of the streptomyces species, and was first isolated by *L J Decaris* in 1947 from *Streptomyces lavendulae* It is related to neomycin in chemical composition (3, 9, 14) in vitro activity (7), and toxicity (7, 1)

Chemically, framycetin is a base composed of neamine, a diaminohexose and a pentose (3, 9 14) Neomycin A is identical with neamine and neomycin B and C are composed of neamine, a diaminohexose and D ribose (4, 14) Thus framycetin is closely related to neomycin B and C and *Rinehart et al* (9) claim that is is identical with neomycin B, proving that the unknown pentose in framycetin (3) is D ribose and that the diaminohexoses in framycetin and neomycin B are identical with an error probably caused by 3 per cent neomycin C in the framycetin Framycetin sulphate is a white powder which is readily soluble in water and has a pH of 5 8 in a 0 23 per cent solution

Generally framycetin is used for local application or is administered perorally in order to reduce the intestinal bacterial flora either preoperatively or in the treatment of infantile gastroenteritis The only report on "

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persons but only traces after sixteen hours, and a resorption of only 3 to 5 per cent after peroral administration

Louvette et al

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mycin treatmen [illegible] they did not observe any toxic effects *Massenat-Deroche* (7) found good results in topical treatment of abscesses [illegible]

[1] The writer is grateful to *Leo Ltd* Copenhagen for supplying the framycetin sulphate

of framycetin against various common pathogenic bacterial groups to work out a method for sensitivity testing of bacterial specimens in routine analysis, and to evaluate any cross resistance between framycetin and streptomycin, neomycin and kanamycin, with particular reference to comparison with neomycin B

MATERIAL AND METHODS

Framycetin was tested as sulphate against 160 bacterial strains belonging to 31 species of 14 genera. These strains consist of a few type cultures and of many strains from routine examination of faeces, urine, sputum and pus from patients. They have been selected so as to be representative of most of the common pathogenic bacteria and have been stored in this laboratory for 3 to 15 years.

The *inoculum* was standardized for the different genera to give a granular semi confluent growth. It was made from a 20 hour culture in 5 per cent serum broth diluted with 0.9 per cent NaCl to the following approximate concentrations: pneumococci 1:100, streptococci except faecalis 1:500, faecal streptococci, staphylococci, gram positive rods, past. pseudotuberculosa 1:3000 and other gram negative rods 1:15 000. One loopful was spread over 1 to 2 cm² in the plate dilution method and 0.1 ml spread with a bent glass rod over 60 cm² in the prediffusion test.

Plate Dilution Method

9 cm petri dishes were filled with 5 per cent blood agar containing framycetin sulphate in two fold dilution steps from 75 µg/ml to 0.3 µg/ml together with control plates without framycetin. The plates were stored at 4° C and used during the subsequent three weeks. Sixteen strains were tested on each plate and the plates were incubated at 35° C for 20 hours. The 50 per cent inhibitory concentration (IC 50) was determined essentially as described by *Reyn et al.* (8) using the method of Kärber. In the present work, however, the rather small inoculum permitted an evaluation of 25, 50–75 per cent reduction in the number of colonies and the IC 50 therefore corresponds to approximately 50 per cent reduction. The minimal inhibitory concentration (MIC) is then assumed to be equal to (1.4–2) times IC 50, except for the strains with a flat dose/response curve, i.e. all the streptococcal and pneumococcal strains, where it is considerably higher.

Agar Prediffusion Method

The method used was the "Method 5" (12) using 6 mm paper discs (Schleicher & Schüll 740 E No 2247) containing 12.5, 25, 50, 100 µg framycetin sulphate and a prediffusion period of 20 hours at room temperature on 10 per cent blood agar plates.

On the basis of these results and the therapeutical framycetin sulphate concentrations available, a conversion scheme has been prepared for use in sensitivity testing.

RESULTS

The number of strains inhibited by the different concentrations is shown in Table 1. Framycetin is active against most of the gram negative rods, staphylococci and some gram positive rods. 95 per cent of the coli, Klebsiella, Salmonella and staphylococcal strains had an IC 50 lower than 1 µg/ml and a steep dose/response curve, ranging from full inhibition to full growth in 2–4 fold dilutions (mainly 2 fold).

These results differ from those obtained with streptococci and pneumococci, 80 per cent of which had an IC 50 higher than 20 µg/ml. These bacteria all had a flat dose/response curve, i.e. a partial growth inhibi-

TABLE 1

	50% inhibitory concentration in µg ml framycetin sulphate							
	≤ 0 2	0 21 – 0 49	0 5 – 1 6	1 7 – 4 9	5 0 – 15 9	16 – 49	50 – 105	> 105
Escherichia coli		3	4	1				
Klebsiella		7	1					
Proteus mirabilis				8				
Proteus morgani			2	6				
Proteus rettgeri		2	2	4				
Proteus vulgaris			1	7				
Providencia			6	2				
Pseudomonas aeruginosa				4	4			
Salmonella		5	2	1				
Shigella			5	3				
Pasteurella pseudotuberculosis		4	2		2			
Bacterium anitratum		1	5	2				
Bacillus cereus et subtilis		1		1				2*
Corynebacterium diphtheriae	4							
Listeria monocytogenes	8							
Staphylococcus aureus		5	3					
Staphylococcus epidermidis	5	3						
Streptococcus non haemolyticus				2*				6
Streptococcus haemolyticus							4	4
Streptococcus faecalis					2	5	1	
Pneumococcus					2	5	1	
Sensitivity groups	+++		++		+		0	

* Verified several times

tion over 4 to 32 fold dilutions, giving an MIC higher than 80 µg ml Of the eight non haemolytic streptococci tested the two which were found moderately sensitive differed from the other six in other respects, being strongly alpha haemolytic and showing a similar sensitivity to kanamycin

Routine Determination of Bacterial Sensitivity to Framycetin

Classification of the bacteria into different sensitivity groups is based on the concentration of the antibiotic in serum after parenteral application The only report available concerning the concentration in human serum (7) states that the value is 1 2 µg ml 8 hours after subcutaneous injection of 250 mg Strains with an IC 50 below one third to one fifth of that value are cons

(+ + +) in Table 1 Strains

to MIC > approximately 100

treatment (0) and the inter ... ivity and resistance has arbitrarily been divided into two sections (+ + +) These same limits between sensitivity groups are indicated by stippled horizontal lines in Fig 1

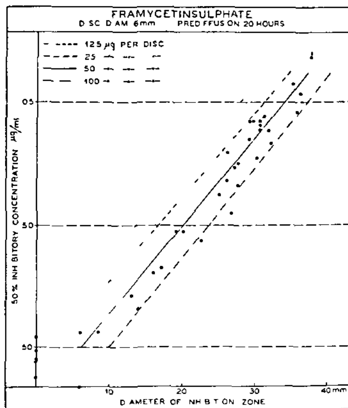


Fig 1

Correlation between values obtained by the plate dilution and prediffusion methods Each point indicates one strain tested twice The horizontal lines show the limits between sensitivity groups

The principle for adjusting the prediffusion test to the plate dilution test will be outlined briefly A number of strains (in this case 37) with *different sensitivity to framycetin were selected from the strains used* in this study The inhibition zones and the IC 50 were determined twice on the same day using the same culture as inoculum in both tests The discs were arbitrarily chosen to contain 12 5–25 50 100 μg framycetin sulphate per disc

Fig 1 shows the results for 50 μg per disc and the regression curve drawn with a continuous line This amount per disc was chosen for sensitivity testing, because it gives inhibition zones approximately *equal* to the disc diameters with strains of IC 50 = 50 μg ml The figure also shows the regression curves for the other three disc strengths, but with- " " For the sake of clearness these have ations were of the same magnitude as those shown

Cross Resistance

Preliminary analysis (2) of the IC 50 for streptomycin (S), kanamycin (K), neomycin (N) and framycetin (F) for bacteria from

TABLE 2

		50% inhibitory concentrations in μg/ml									
		E. coli	E. coli	Klebsiella	S. typhimurium	S. enteritidis	Sh. boy III	Sh. flexneri	Sh. boydii	P. morganii	Prot. densl.
Original strains	S	1 6	1 9	>50	2 5	1 6	3 0	0 8	3 1	>50	1 9
	K	0 8	1 8	0 9	1 7	2 2	2 3	0 8	2 2	1 1	3 1
	N	1 4	2 1	1 6	1 8	1 9	1 8	1 1	2 8	3 4	2 6
	F	1 0	1 4	1 1	1 1	1 1	1 6	1 0	2 1	1 3	2 5
Strains after five passages in framycetin broth	S	2 9	8 3	>50	50	11	2 5	1 0	1 9	>50	3 5
	K	4 0	>50	1 9	>50	5 8	4 2	1 4	3 3	10	3 8
	N	2 8	12	0 9	9 4	4 6	3 4	2 1	1 5	5 5	3 4
	F	2 2	13	0 8	8 2	7 0	2 6	2 2	1 2	5 0	2 6
Strains after five passages in neomycin B broth	S	5 8	7 0	>50	>50	7 0	3 1	1 0	5 0	>50	2 9
	K	>50	50	3 3	>50	17	4 1	2 5	7 2	20	4 4
	N	22	23	2 8	38	15	2 1	4 7	5 5	22	18
	F	19	24	1 3	22	8 2	2 1	2 2	4 5	26	16

S — streptomycin K — kanamycin N — neomycin B F — framycetin

wounds, using the prediffusion technique, shows the following pattern when differences in IC 50 larger than 10 fold are recorded Of the 128 strains, 39 were S resistant and K N F sensitive (mainly staphylococci), 2 were S K resistant and N F sensitive, and 3 *streptococcus faecalis* were K resistant and S N F moderately sensitive However, in no case was any difference found between N and F

In order to investigate the possible cross resistance more thoroughly, 8 S K N F sensitive and 2 S resistant K N F sensitive gram-negative rods were grown in five passages in dilution rows in filtered broth with framycetin sulphate or neomycin B sulphate in concentrations from 0 2–48 6 μg/ml, each time taking the inoculum from the last tube giving dense growth The results are shown in Table 2 The IC 50 for all 10 strains was measured before the *in vitro* development of resistance and after five passages in framycetin sulphate broth or neomycin sulphate broth

It will be seen that 6 of the 10 strains developed *in vitro* resistance and that the rise in N F resistance was of the same order for all these strains when made resistant to neomycin B alone or to framycetin alone, whereas the increase in S or K resistance differed more than 5 times from N F in the *S typhimurium* and the *Providencia*

DISCUSSION

When choosing antibiotics for use in the treatment of bacterial infections, the clinician must know the spectrum, the therapeutical concentrations available, the toxicity and the degree of development of resistance of the antibiotic substances in question

The spectrum shown in Table 1 is in good agreement with the findings of *Massenat-Deroche* (7) and *Maccabe* (6), but rather different from those of *Fairbrother et al* (1) The latter authors report MIC values for *Staphylococcus aureus* and *Proteus* which are 20 to 50 times higher, and for *Pneumococcus* 10 to 25 times lower, than those in the present study *The reason for this is not clear, since the dose/response* curve in this study is quite steep for all the staphylococcal and proteus species tested and very flat for the pneumococci This should influence the results in the opposite direction However, one explanation may be that in the work of *Fairrother et al* (1) the inoculum was rather heavy *i e* approximately 10^7 to 10^8 staphylococci or proteus, in contrast to 10^3 to 10^4 in the present work

Up to now, only one report exists regarding serum concentrations and toxicity in man, *viz* the one by *Massenat-Deroche* (7), who stated that 0 25 g framycetin given subcutaneously give a mean *serum concentration* in six persons of 1 2 μg ml after 8 hours and nothing after 16 hours On that basis, 0 75 to 1 0 g was given per day, but after administration for some weeks that dose caused lesions of the eight cranial nerves and renal filtration impairment similar to the condition

found after parenteral treatment with streptomycin and neomycin For that reason, framycetin has seldom or never been used parenterally since then

However, it has been used in local treatment in cases of infantile gastroenteritis (5, 10) and of various wounds (7), with results which were comparable to those after treatment with neomycin In the treatment of wounds it must be borne in mind that framycetin is not active against most streptococci

In vitro resistance was found to develop both against neomycin B and framycetin—possibly slight faster to the former than to the latter In both cases there was 100 per cent cross resistance between these two substances, whereas in two cases there was a large difference as regards kanamycin and streptomycin Furthermore, the regression curve (Fig 1) is practically identical with the neomycin B regression curve but differs significantly in slope from the streptomycin and kanamycin curves made with the same technique (13) These facts further support the assumption of the identity of neomycin B and framycetin which has already been proposed on the basis of chemical analysis (9)

SUMMARY

The plate dilution method has been used to determine the *in vitro* activity of framycetin against 160 bacterial strains belonging to 14 genera Framycetin is active against most gram negative rods and staphylococci but has a low activity against pneumococci and many streptococci

Based on the serum concentrations obtained, the bacteria have been classified into sensitivity groups A prediffusion test has been worked out for use in special cases, while the sensitivity test with neomycin B is considered adequate for the daily routine work

Cross resistance between framycetin and neomycin B has been found in all the strains tested The question of the identity of these two substances is considered, and the chemistry is reviewed on the basis of the literature

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FROM THE TUBERCULOSIS AND STATISTICAL DEPARTMENTS
STATENS SERUMINSTITUT COPENHAGEN DENMARK
AND THE NATIONAL INSTITUTE OF HEALTH SEOUL KOREA

TUBERCULIN PRODUCTION

5 *Relationship between Age of Culture of Mycobacterium tuberculosis and Tuberculin Activity of Culture Filtrate*

By

Mogens Magnusson, Hyun Kyu Kim[1] and M Weis Bentzon

Received 23 ix 63

The age of the cultures of *Mycobacterium tuberculosis* used for tuberculin production varies in different laboratories from four to twelve weeks (*Calmette & de Potter* 1926, *Seibert & Glenn* 1941, *Green* 1946, *McIntosh & Konst* 1947, *Lind* 1947 1948 *Baisden, Larsen & Vardaman* 1955 *Svenkerud* 1955, *Desbordes* 1958, *Magnusson & Bentzon* 1958, *Ministry Hlth Welf*, *Jap* 1961)

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& *Bentzon* 1963) or *yield of tuberculin* obtained from the filtrate (*Seibert* 1928 *Lind* 1948, *Shiraishi* 1951, *Asami et al* 1953c, d) has been studied previously Studies of filtrates of *M bovis* (*Boquet & Bretey* 1933 *Lind* 1948 *Asami et al* 1953b) and *M paratuberculosis* (*Johnson* 1944) have also been reported Others (*Seibert & Munday* 1932, *Wong* 1937 *Corper Cohn & Bower* 1940, *Paterson* 1948 *Svenkerud* 1955, *Magnusson Kim & Bentzon* 1963a) have investigated the yield of tuberculoprotein in relation to the age of the culture without measurement of the tuberculin activity

The findings vary considerably Usually the tuberculin activity of the filtrate (*Boquet & Bretey* 1933 *Shiraishi* 1951, *Asami et al* 1952, 1953a *Baisden, Larsen & Vardaman* 1955) or the yield of tuberculin (*Asami et al* 1953c d) increases with the age of the culture up to a certain time after which it remains constant However, *Lind* (1948) and *Sawada et al* (1955) have found a decrease in the yield of tuberculin for old cultures

The incubation period required for obtaining maximum tuberculin activity or yield of tuberculin has varied from four weeks (*Sawada et al* 1955) five (*Shiraishi* 1951), five or more (*Lind* 1948) to eight (*Asami*

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[1] Present address Department of Medical Microbiology The University of Wisconsin Madison 6 Wisconsin USA

et al 1953b) or eleven weeks (*Baisden, Larsen & Vardaman* 1955). Apparently the composition of the medium (*Lind* 1948, *Asami et al* 1953a, d, *Sawada et al* 1955), the strain and especially its growth rate (*Lind* 1948, *Asami et al* 1953a, d, *Kim, Magnusson & Bentzon* 1963), and the preparation method of the tuberculin (*Lind* 1948) have an effect on the time the tuberculin activity of the culture filtrate attains its maximum. The study population in which the biological assay is performed may possibly also be of significance (*Asami et al* 1952, 1953a, d).

The purpose of the present work is to study the tuberculin activity in BCG vaccinated guinea pigs of heat-sterilized culture filtrates of *M. tuberculosis* of varying ages, and its relationship to the tuberculo protein content of the filtrates under the same conditions as used in other studies in the present series (*Magnusson, Kim & Bentzon* 1963a, b, *Kim, Magnusson & Bentzon* 1963, 1964a).

EXPERIMENTAL

In a previous study (*Magnusson, Kim & Bentzon* 1963b) two strains of *M. tuberculosis*, T3505 and PN, were cultured at 38° C for two to nine weeks in flasks containing 180 ml Lind bII medium (*Lind* 1948). The cultures were sterilized in streaming steam (105° C) for one hour and the dry weight of organism, the tuberculo protein content and the pH of the culture filtrate measured (*Magnusson, Kim & Bentzon 1963b*). *In the present work the tuberculin activity of these culture filtrates* has been measured by skin tests on BCG vaccinated guinea pigs.

Experimental animals. Twenty four female albino guinea pigs bred at the farm attached to Statens Seruminstitut and weighing 450–570 g were vaccinated intracutaneously in four sites on the abdomen with 0.1 ml of BCG vaccine containing 3.0 mg/ml and prepared at the BCG Department, Statens Seruminstitut.[1] The vaccines (batches Nos. 1152 and 1154) were used when freshly prepared. The animals were used two to four weeks after the vaccination.

Experimental design. The eight culture filtrates of strain T3505 were injected together with dilutions containing 100 tuberculin units (TU) and 10 TU per 0.1 ml on to a group of six guinea pigs (study A). Before injection the filtrates were diluted 1:4, 1:20 or 1:200 using phosphate buffered saline pH 7.38 as diluent. Purified tuberculin (PPD) RT 22, Statens Seruminstitut, was used as standard tuberculin; 1 mg of RT 22 was assumed to be equal to 75 000 TU. The ten injections were allocated at random to ten test sites on the sides and backs of the guinea pigs.

Similar tests were made with the culture filtrates of strain PN on another group of six guinea pigs (study B). In study C culture filtrates of 6–9 weeks old cultures of the two strains were injected into a group of 12 guinea pigs together with the two standard dilutions.

Injection and reading. The technique for injection and reading of the reactions [illegible] (*Kim, Magnusson & Bentzon* 1963). The volume [illegible]

[illegible] n activity D—in TU per ml of culture filtrate— [illegible] of reactions according to the method published [illegible] t-on 1963b).

Statistical [illegible] al analysis of the variability of the results is presented in Appendix 1.

RESULTS

The tuberculin activity of the culture filtrates of strain T3505, estimated on the basis of the readings after 24 hours (Table 1), increased

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[1] [illegible]

TABLE 1

Mean Size of Intradermal Reactions after 24 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrate of 2 to 9 Week Old Cultures of M tuberculosis strain T3505 on F and bH Medium

Age of culture (weeks)	Study A			Study C			Tuberculin activity (study A+C) (TU ml)
	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	
2	1 4	16 4 (6)	1 700				1,700
3	1 20	16 5 (6)	9 200				9 200
4	1 20	17 8 (6)	21 000				21 000
5	1 200	15 8 (6)	59 000				59 000
6	1 200	15 7 (6)	56 000	1 200	14 3 (12)	46 000	49 000
7	1 200	16 4 (6)	86 000	1 200	15 5 (12)	98 000	94 000
8	1 200	16 4 (6)	86 000	1 200	16 3 (12)	100 000	130 000
9	1 200	17 4 (6)	160 000	1 200	16 5 (12)	180 000	175 000

Mean reaction to standard dilution Study A 100 TU 17 7 mm (6) 10 TU 14 1 mm (6)
Mean reaction to standard dilution Study C 100 TU 16 7 mm (12) 10 TU 12 9 mm (12)

Figures in brackets indicate number of reactions

et al 1953b) or eleven weeks (*Baisden, Larsen & Vardaman* 1955) Apparently the composition of the medium (*Lind* 1948, *Asami et al* 1953a, d, *Sawada et al* 1955), the strain and especially its growth rate (*Lind* 1948, *Asami et al* 1953a, d, *Kim, Magnusson & Bentzon* 1963), and the preparation method of the tuberculin (*Lind* 1948) have an effect on the time the tuberculin activity of the culture filtrate attains its maximum The study population in which the biological assay is performed may possibly also be of significance (*Asami et al* 1952, 1953a, d)

The purpose of the present work is to study the tuberculin activity in BCG vaccinated guinea pigs of heat-sterilized culture filtrates of *M tuberculosis* of varying ages, and its relationship to the tuberculo-protein content of the filtrates under the same conditions as used in other studies in the present series (*Magnusson, Kim & Bentzon* 1963a, b, *Kim, Magnusson & Bentzon* 1963, 1964a)

EXPERIMENTAL

In a previous study (*Magnusson Kim & Bentzon* 1963b) two strains of *M tuberculosis* T3505 and PN were cultured at 38° C for two to nine weeks in flasks containing 180 ml Lind bII medium (*Lind* 1948) The cultures were sterilized in streaming steam (105° C) for one hour and the dry weight of organism the tuberculo protein content and the pH of the culture filtrate measured (*Magnusson Kim & Bentzon* 1963b) In the present work the tuberculin activity of these culture filtrates has been measured by skin tests on BCG vaccinated guinea pigs

Experimental animals Twenty four female albino guinea pigs bred at the farm attached to Statens Seruminstitut and weighing 450 570 g were vaccinated intracutaneously in four sites on the abdomen with 0 1 ml of BCG vaccine containing 3 0 mg/ml and prepared at the BCG Department Statens Seruminstitut [1] The vaccines (batches Nos 1152 and 1154) were used when freshly prepared The animals were used two to four weeks after the vaccination

Experimental design The eight culture filtrates of strain T3505 were injected together with dilutions containing 100 tuberculin units (TU) and 10 TU per 0 1 ml on to a group of six guinea pigs (study A) Before injection the filtrates were diluted 1 4 1 20 or 1 200 using phosphate buffered saline pH 7 38 as diluent Purified tuberculin (PPD) RT 22 Statens Seruminstitut was used as standard tuberculin 1 mg of RT 22 was assumed to be equal to 75 000 TU The ten injections were allocated at random to ten test sites on the sides and backs of the guinea pigs

Similar tests were made with the culture filtrates of strain PN on another group of six guinea pigs (study B) In study C culture filtrates of 6 9 weeks old cultures of the two strains were injected into a group of 12 guinea pigs together with the two standard dilutions

Injection and reading The technique for injection and reading of the reactions [illegible] 1963) The volume [illegible]

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RESULTS

The tuberculin activity of the culture filtrates of strain T3505, estimated on the basis of the readings after 24 hours (Table 1), increased

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TABLE 2

Mean Size of Intradermal Reactions after 24 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrate of 2 to 9 Week Old Cultures of M tuberculosis strain PN on F and bH Medium

Age of culture (weeks)	Study B			Study C			Tuberculin activity (study B+C) (TU/ml)
	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	
2	1 20	15 7 (6)	5 900				5 900
3	1 20	17 8 (6)	22 000				22 000
4	1 200	15 2 (6)	43 000				43 000
5	1 200	15 6 (6)	56 000				56 000
6	1 200	16 6 (6)	105 000	1 200	16 1 (12)	140 000	130 000
7	1 200	17 0 (6)	115 000	1 200	15 9 (12)	125 000	130 000
8	1 200	17 2 (6)	150 000	1 200	15 4 (12)	92 000	110 000
9	1 200	16 7 (6)	110 000	1 200	15 9 (12)	125 000	120 000

Mean reaction to standard dilution Study B 100 TU 17 6 mm (6) 10 TU 14 0 mm (6)
Mean reaction to standard dilution Study C 100 TU 16 7 mm (12) 10 TU 12 9 mm (12)

Figures in brackets indicate number of reactions

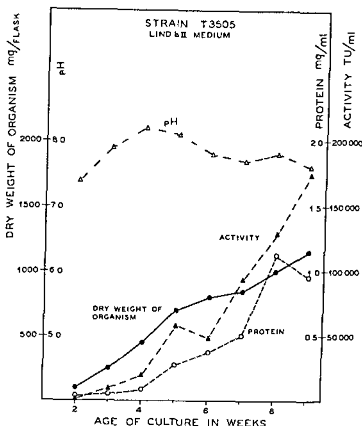


Fig 1

Dry weight of organism (●), pH (△), protein content (○) and tuberculin activity (▲) of culture filtrate of *Mycobacterium tuberculosis* strain T3505 cultured on Lind bII medium in relation to age of culture

with the age of the culture from 1,700 TU/ml in 2-week-old to 175,000 TU/ml in 9-week old cultures This is illustrated in Fig 1 which also shows the relation between the age of the culture and the bacterial crop, the protein content and the pH of the filtrates [1] The bacterial crop and the protein content of the culture filtrate also increased with the age of the culture, reaching maximum values of 1100 mg of dried culture per flask and 1 0–1 1 mg of protein per ml, respectively The pH increased to 8 2 (4-week-old culture) and thereafter decreased to 7 6 (9-week-old culture)

The activity of the culture filtrate of strain PN increased with the age of the culture from 5,900 TU/ml at two weeks to 130,000 TU/ml in 6-week-old cultures (Table 2) There were no significant changes in the activity from the 6th to the 9th week (Table 2) The dry weight of organism was maximal (2050 mg per flask) after seven weeks' culture (Fig 2 and Appendix 2) The protein content of the filtrate was maximal—0 55 mg/ml—after 6 weeks, and had decreased to 0 28 mg/ml after

[1] The bacterial crop, the protein content and pH of the culture filtrates of both strains are also shown in Appendix 2

TABLE 3

Mean Size of Intradermal Reactions after 48 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrate of 2 to 9 Week Old Cultures of M tuberculosis strain T3505 on I and bII Medium

Age of culture (weeks)	Study A			Study C			Tuberculin activity (study A+C) (TU ml)
	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	
2	1 4	13 9 (6)	1 800				1 800
3	1 20	13 7 (6)	8 100				8 100
4	1 20	14 4 (6)	12 000				12 000
5	1 200	12 6 (6)	47 000				47 000
6	1 200	13 8 (6)	85 000	1 200	11 6 (12)	53 000	62 000
7	1 200	13 8 (6)	85 000	1 200	12 5 (12)	83 000	84 000
8	1 200	14 4 (6)	115 000	1 200	13 2 (12)	120 000	115 000
9	1 200	14 7 (6)	135 000	1 200	13 6 (12)	145 000	140 000

Mean reaction to standard dilution Study A 100 TU 15 3 mm (6), 10 TU 11 1 mm (6)
Mean reaction to standard dilution Study C 100 TU 14 2 mm (12) 10 TU 9 7 mm (12)

Figures in brackets indicate number of reactions

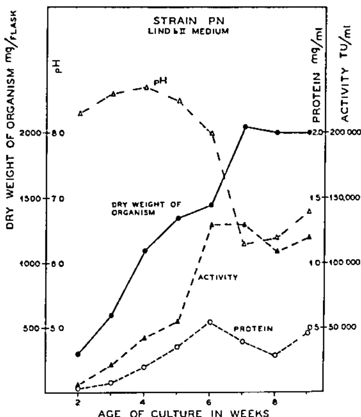


Fig 2

Dry weight of organism (●), pH (△) protein content (○) and tuberculin activity (▲) of culture filtrate of *Mycobacterium tuberculosis* strain PN cultured on Lind bII medium in relation to age of culture

eight weeks' culture (Fig 2) The pH of the culture filtrate increased to 8 7 (4-week-old culture) and thereafter decreased to 6 3 (7-week-old culture) (Fig 2)

In general, the mean reactions decreased by 2 to 3 mm from 24 to 48 hours (Tables 1 to 4) The 48 hours observations indicate that the activity of the seven-week-old filtrate of strain PN (99,000 TU ml, Table 4) is smaller than that of the six-week-old filtrate (155,000 TU/ml) Such a decrease was not found on the basis of the 24 hours readings (Table 2) With this exception, the results after 48 hours (Tables 3 and 4) show the same relationship as after 24 hours (Tables 1 and 2)

Relationship between protein content and tuberculin activity The logarithms of the amount of protein (in mg per ml) and the tuberculin activity (in TU per ml) of the culture filtrates have been calculated and the results are shown graphically in Fig 3 With the exception of the result for one single filtrate of strain T3505—a two week-old culture the points seem to be located on a line with slope 1 This means that the activity per mg of protein is fairly constant—about 200,000 TU for

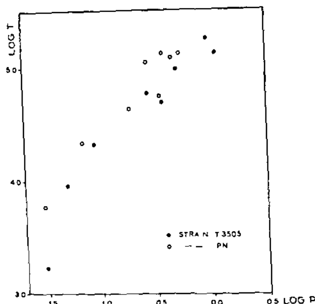


Fig 3

Tuberculin activity in relation to protein content of culture filtrates of *Mycobacterium tuberculosis* cultured on Lind bII medium
Abscissa Logarithm of protein content in mg per ml culture filtrate
Ordinate Logarithm of tuberculin activity in TU per ml culture filtrate

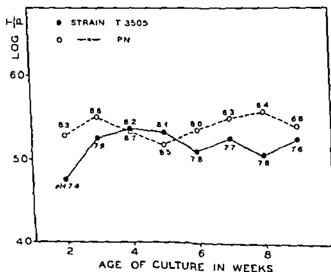


Fig 4

Tuberculin activity of tuberculoprotein from culture filtrates of *Mycobacterium*

TABLE 4

Mean Size of Intradermal Reactions after 48 Hours (Erythema in mm) of BCG Vaccinated Guinea Pigs and Tuberculin Activity (in Tuberculin Units per ml) of Culture Filtrate of 2 to 9 Week Old Cultures of M tuberculosis strain PN on I and bII Medium

Age of culture (weeks)	Study B			Study C			Tuberculin activity (study B+C) (TU ml)
	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	Dilution	Mean reaction (mm)	Tuberculin activity (TU ml)	
2	1:20	13.9 (6)	13 000				13 000
3	1:20	15.7 (6)	31 000				31 000
4	1:200	12.9 (6)	77 000				77 000
5	1:200	13.6 (6)	110 000				110 000
6	1:200	14.3 (6)	155 000	1:200	13.7 (12)	150 000	155 000
7	1:200	13.3 (6)	94 000	1:200	12.9 (12)	100 000	99 000
8	1:200	14.7 (6)	190 000	1:200	13.0 (12)	105 000	130 000
9	1:200	15.0 (6)	220 000	1:200	13.6 (12)	145 000	165 000
Mean reaction to standard dilution Study B	100 TU	15.1 mm (6)		10 TU	9.9 mm (6)		
Mean reaction to standard dilution Study C	100 TU	14.2 mm (12)		10 TU	9.7 mm (12)		

Figures in brackets indicate number of reactions

tures The relative activity increased with decreasing pH (pH <8) and had a pronounced maximum at about pH 4

The residual error (see appendix 1) is slightly smaller in the present study than in a previous study (*Magnusson, Kim & Bentzon* 1963b) and appears to be smaller in the present studies A and B than in C This is probably due to the tuberculin preparations being more alike in studies A and B (cultures of different age of one strain) than in C (two different strains) or in the previous study (several different strains of varying ages)

The present study indicates that the tuberculin activity of the culture filtrate will increase as long as the bacterial crop increases, and that, for a given strain of *M tuberculosis* cultured on a given medium, the tuberculin activity of the filtrate will increase proportionally to the tuberculoprotein content, provided only minor variations in the pH of the filtrates take place

SUMMARY

Two strains of *Mycobacterium tuberculosis* were cultured at 38° C for two to nine weeks on Lind bII medium and the tuberculin activity of the culture filtrates of heat sterilized cultures measured by intradermal tests on BCG vaccinated guinea pigs

The activity of the filtrate of one strain increased with the age of the culture up to nine weeks, when it was 2–3 times larger than in a 5 week old culture The activity of the filtrate of the other strain increased up to six weeks, after which it hardly changed

The tuberculin activity per mg of tuberculoprotein was fairly constant in the present study

APPENDIX 1

The mean reactions for the different tuberculins are based on 6 or 12 single reactions read after 24 and 48 hours each time by two readers The variances due to different sources have been estimated by analysis of variance and are shown in Appendix Table 1

APPENDIX TABLE 1

Analysis of Variance of Intradermal Reactions of BCG Vaccinated Guinea Pigs Read after 24 and 48 Hours

Nature of variation	Study A n = 6			Study B n = 6			Study C n = 12			Average		
	Number of animals											
	df*	Mean square		df*	Mean square		df*	Mean square		df*	Mean square	
		24 hrs	48 hrs		24 hrs	48 hrs		24 hrs	48 hrs		24 hrs	48 hrs
Reading error	45	0.42	0.62	45	0.53	0.66	99	0.51	0.74	189	0.49	0.69
Residual between animals	45	0.71	1.06	45	0.60	0.94	99	0.91	1.21	189	0.79	1.11

* df indicates degrees of freedom

the culture filtrates It appears to be higher (275,000 TU) for strain PN than for T3505 (160,000 TU).

The activity per mg of protein would not seem to be dependent on the age of the culture or the pH of the culture filtrate (Fig 4).

DISCUSSION

In the present study, the tuberculin activity of the heat-sterilized culture filtrates of both strains increased with the age of the culture in the period during which the bacterial crop increased

Beyond that period only one of the strains (PN) was observed and no further increase in the activity of the filtrates was found On the other hand, also the bacterial crop remained constant and thus there was no evidence of autolysis of the cultures Therefore the significance of autolysis for the formation of tuberculin (*Corper & Cohn* 1943, 1944, *Paterson* 1948, *Green* 1953, *Svenkerud* 1955, *Magnusson, Kim & Bentzon* 1963a) can hardly be evaluated on the basis of the present study

From a practical point of view, it would appear from the present study that cultures of *M tuberculosis* used for tuberculin production should preferably be incubated only as long as bacterial growth takes place This period varies with the conditions of culture (medium, strain, etc)

In some previous studies, the tuberculin activity of culture filtrates (*Asami et al* 1952) or the yield of tuberculin (*Asami et al* 1953a, c, d) was measured by simultaneous skin tests on humans and guinea pigs using cultures of different ages In tests on *humans*, the activity seemed to be maximal at the same time as the bacterial crop, whereas on *guinea pigs* it was at its height several weeks later in the majority of the studies (*Asami et al* 1952, 1953a, d) Thus the results of the present study, where the activity was measured on BCG vaccinated guinea pigs, are in agreement with the results obtained in humans by *Asami et al* (1952, 1953a, d) but not with the results the same authors obtained in guinea pigs

The tuberculin activity *per mg of tuberculoprotein* is fairly constant in the present study, although it appears to be different for the two strains (275,000 TU for PN and 160,000 TU for T3505) Similar results have been reported previously by *Takehara* (1957) Apparently the tuberculin activity of the protein is not dependent on the age of the culture or the pH of the culture filtrate in the present study The tuberculoprotein content of the filtrates can therefore be used as a measure of their tuberculin activity However, this is not generally the case (*Kim, Magnusson & Bentzon* 1963) In the present study, the pH varied only between 6 3 and 8 7 In a subsequent study (*Kim, Magnusson & Bentzon* 1964b) the pH of culture filtrates of *M tuberculosis* has been varied deliberately within a wider range by adding hydrochloric acid or sodium hydroxyde before heat sterilization of the cul-

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The average mean squares 0.79 (24 hrs.) and 1.11 (48 hrs.) are used for the calculation of the standard errors given below.

APPENDIX TABLE 2

Standard Errors of Mean Reactions and Tuberculin Activities (D)

Standard Error	Study A and B n = 6		Study C n = 12		Study A+C and B+C n = 18	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
Mean reactions	0.36	0.43	0.26	0.30	0.21	0.25
Log D	0.120	0.114	0.085	0.081	0.070	0.066
Difference between 2 log D values same experiment	*0.139§*	*0.132§*	*0.098*	*0.094*	*0.081*	*0.076*

§ Valid for comparison between log D values for a 2.5 weeks' and a 6.9 weeks old culture.

From the standard errors (S.E.) of log D, 95 per cent confidence limits of the tuberculin activity are obtained as D × antilog (2 × S.E. of log D). Furthermore, standard errors of the difference between two values of log D found in independent experiments can be calculated in the usual way. However, when comparing two D values within the same experiment, it must be taken into account that average reactions to the standard tuberculins are used for calculation of both values. The appropriate standard errors of the difference between two log D values are given in the last line of Appendix Table 2.

APPENDIX 2

*Bacterial Crop (in mg per Flask), pH and Tuberculoprotein Content (in mg/ml) of Heat Sterilized Culture Filtrate of Two Strains of Mycobacterium tuberculosis Cultured on Lind bH Medium in Relation to Age of Culture**

Age of culture (weeks)	Strain T30r: Dry weight of organism (mg)	Strain T30r: Culture filtrate: pH	Strain T30r: Culture filtrate: Tuberculoprotein (mg/ml)	Strain PN: Dry weight of organism (mg)	Strain PN: Culture filtrate: pH	Strain PN: Culture filtrate: Tuberculoprotein (mg/ml)
2	100	7.4	0.03	300	8.3	0.03
3	250	7.9	0.05	600	8.0	0.07
4	450	8.2	0.09	1100	8.7	0.19
5	700	8.1	0.28	1350	8.5	0.36
6	800	7.8	0.38	1450	8.0	0.55
7	850	7.7	0.51	2050	6.3	0.39
8	1000	7.8	1.13	2000	6.4	0.28
9	1150	7.6	0.96	2000	6.8	0.46

* Adapted from *Magnusson, Kim & Bentzon* 1963 b.

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FROM STATENS SERUMINSTITUT COPENHAGEN (DEPARTMENT OF DIAGNOSTIC BACTERIOLOGY)

EPIDEMIC SPREAD OF STAPHYLOCOCCUS AUREUS PHAGE-TYPE 83A

By

KIRSTEN ROSENDAL and OVE JESSEN

Received 24 ix 63

The first occurrence of *Staphylococcus aureus* phage type 83A in England is mentioned by *Williams et al* (1960), and *Williams & Jevons* (1961) report that this type caused 7 and 9 per cent of staphylococcal epidemics in England in 1958 and 1959 respectively

At present Danish hospitals are experiencing a fast spread of the same type Since April 1st 1960 the typing phage 83A has been used routinely in Denmark and at the same time a country wide registration of phage typed hospital staphylococci began at Statens Seruminstitut The simultaneous initial epidemic occurrence of type 83A gave us our first opportunity to follow the distribution of a new epidemic type since type 80—previously the dominating type in hospital epidemics—was already established as an epidemic type when Statens Seruminstitut began routine phage typing in 1955

MATERIAL AND METHODS

All strains have been isolated from patients or staff members and have been phage typed at Statens Seruminstitut which serves as typing laboratory for the whole country Only the first isolated strain from each person is included

Phage typing antibiotic sensitivity tests and tween reaction (egg yolk reaction) were performed as described by *Rosendal et al* (1963) Strains isolated in 1962 and 1963 were examined for resistance to mercuric chloride (*Moore* 1960) as described by *Jessen et al* (1963)

RESULTS

Bacteriological Properties

Most often the type 83A strains are resistant to penicillin streptomycin and—unlike most other strains in Denmark—to tetracyclines (Table 1)

Through the years a fairly constant proportion of about 5 per cent of the strains were sensitive to all antibiotics The relative incidence of multiple resistant strains (penicillin streptomycin tetracyclines) seems to decrease slightly along with a rise in the number of strains resistant to penicillin only

TABLE 1
Antibiotic Resistance of Type 83A Strains

Year	Number of 83A strains	Percentage of strains resistant to				
		Penicillin	Streptomycin	Tetracyclines	Chloramphenicol	Erythromycin
1960*	(242)	89	94	90	4	6
1961	732	92	89	85	4	7
1962	935	87	88	84	4	9
1963§	(670)	94	83	81	6	5

Only the first isolated strain from each patient in included
* last 9 months only
§ first 6 months only

Concordant with previous studies on the tween or egg yolk reaction (*Gillespie & Alder* 1952, *Jessen et al* 1959) and the mercury resistance (*Moore* 1960, *Jessen et al* 1963) these multiple resistant 83A strains are mainly tween negative (81 per cent) and mercury resistant (91 per cent), but to an even greater extent than are other types with the same antibiotic pattern (46 per cent tween negative, 79 per cent mercury resistant)

Spread of Type 83A

Type 83A made up 3 per cent of all phage typed strains in 1960, 6 per cent in 1961, 7 5 per cent in 1962, and 10 6 per cent in the first six months of 1963 In 1960 only seven hospitals had more than five infections due to this type, and its sporadic occurrence was recorded in 29 hospitals (Table 2)

TABLE 2
Type 83A in Danish Hospitals

Number of hospitals with	1960	1961	1962
0 cases	34	24	18
1–5 cases	29	46	46
6–25 cases	4	13	23
26–50 cases	3	3	6
51–100 cases		4	3
> 100 cases	–		1

The table comprises hospitals which have referred more than 10 *Staphylococcus aureus* strains to Statens Seruminstitut per year

It is now found in the majority of hospitals and causes serious epidemics in several departments, especially in surgical wards

The number of cases diagnosed per month is now about 100 as against 10–15 in April-June 1960

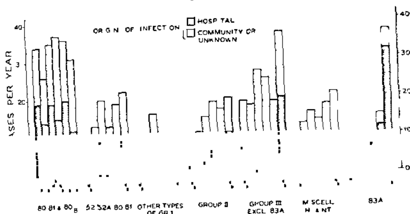


Fig 1

Phage types of 736 *Staphylococcus aureus* strains from bacteraemia cases

Bacteraemia Due to Type 83A

By retyping strains from bacteraemia cases we have traced the type back to 1957 The phage-types of 736 bacteraemia strains are given in Fig 1

By means of clinical and anamnestic evidence hospital infections have been distinguished from infections of out-hospital or uncertain origin

In the six-year-period mentioned 66 cases of bacteraemia were caused by type 83A In 55 cases the hospital origin of the infection was certain The infection was fatal in 45 cases, *i e* the same very high mortality rate as that recorded for corresponding infections due to other staphylococcal types with the same antibiotic resistance and tween reaction, but much higher than the average mortality (about 40 per cent) for the total material

The increasing occurrence of type 83A influences the general sensitivity status of *Staphylococcus aureus*, especially regarding tetracycline resistance For the 736 strains from bacteraemia cases this fact is illustrated in Fig 2

If cases due to type 83A are disregarded, the percentage of tetracycline resistant strains (Fig 2, dotted curve) would be fairly constant, about 10 per cent through the years, corresponding to the percentage found in a larger Danish material (*Rosendal et al* 1963)

In the bacteraemia material the addition of type 83A increases the percentage to 31 and if only hospital infections are regarded even to 46

Occurrence of a New Type Related to 83A

Recently a large number of strains resistant to erythromycin as well as to penicillin, streptomycin and tetracyclines have been isolated These strains have also mercury resistance and negative tween reaction as common characteristics

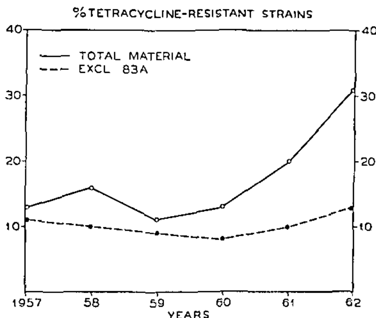


Fig 2

Incidence of tetracycline resistance among 736 *Staphylococcus aureus* strains from bacteraemia cases

The strains cannot be typed with the international set of diagnostic phages, but are lysed by a phage 6557, isolated at Statens Seruminstitut in April 1963 and used routinely since then During the following three months the type was found in about 50 different hospitals A retyping of all previously non-typable strains from the 736 bacteraemia cases during the years 1957–62 revealed 17 cases due to the new type, and 17 cases were diagnosed during the first six months of 1963

Studies which will be reported elsewhere (*Bulow & Rosendal*) suggest that the type is intimately related to type 83A

DISCUSSION

Through the last 15 years the following types have successively prevailed as epidemic staphylococci in Denmark Types 3A and 29A 44 in most cases sensitive to antibiotics (*Andersen* 1950), type 80, most often resistant to penicillin and streptomycin, type 83A, resistant to tetracycline as well, and recently a new type furthermore resistant to erythromycin The emergence of resistant types has, with a few years delay, followed the introduction for clinical use of the antibiotic concerned

On this evidence alone it might be tempting to postulate that certain types more easily than others acquire resistance to a given antibiotic, and that the selection of such strains due to the use of new antibiotics determines the shift in prevalent epidemic types As applied to the spread of type 83A in Denmark the theory would thus imply a marked

selection of tetracycline resistant strains However, it is evident that the theory is inadequate, at it leaves unexplained the facts that no significant spread is recorded for tetracycline resistant strains of other types (Fig 2), and that tetracycline *sensitive* type 83A strains seem to spread at an even faster rate than the resistant ones do, although the absolute figures are smaller (Table 1)

Consequently the epidemic character of certain types must be due primarily to other properties (summarized as "communicability") that vary from type to type and to a certain extent may be acquired or lost Instead of a causal relation between antibiotic resistance and epidemic spread we must sooner assume a parallelism between these properties, the strains likely to acquire resistance against antibiotics in current use being those which for the moment also have a high grade of communicability These two properties reflect the ability of the organism to resist the influence of changing environmental conditions, including human host factors Both of these may be due to a high mutation rate or a pronounced adaptability regarding a number of enzymatic systems, or to the possession of several alternate essential metabolic pathways An example of one property which seems related to communicability is mercury resistance, although its possible rôle is unknown

It has been suggested that a particular tendency to cause long-standing discharging cutaneous infections was decisive for the spread of type 80 In a large Danish material from 1960, 50 per cent of boils and pustules were caused by type 80, whereas as few as 22 out of 1730 cases (i e, 1 3 per cent) were caused by type 83A (*Rosendal et al* 1963) This percentage had increased to 3 4 in 1962 These figures do not suggest that cutaneous infections are main factors in the initial rapid spread of the type

A possible experimental approach to the question of communicability would be an investigation of mutation rates regarding a number of bacterial properties including antibiotic resistance, for epidemic and non epidemic strains, and for newly established epidemic types as compared with the same types towards the decline of the epidemic

SUMMARY

During the last three years an epidemic spread of *Staphylococcus aureus* type 83A has occurred in Danish hospitals The type is now the most common cause of hospital acquired staphylococcal bacteraemia The strains differ from most other staphylococci in Denmark in their high rate of tetracycline resistance

The concept of communicability is discussed The antibiotic resistance alone cannot explain the spread of the strain, neither can any special ability to cause cutaneous infections

The occurrence of a related erythromycin resistant type, susceptible to a newly isolated phage, is reported

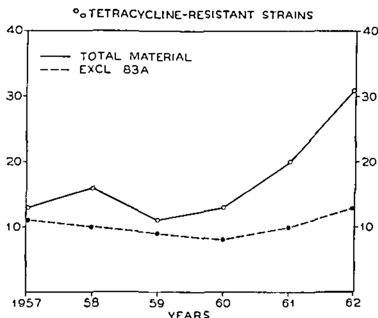


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FROM THE RICKETTSIA AND VIRUS DEPARTMENT,
STATENS SERUMINSTITUT, COPENHAGEN

STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

3 *Duration and Maximal Effect of Adoptive Immunization of Virus Carriers*

By

MOGENS VOLKERT and JØRGEN HANNOVER LARSEN

Received 1 x 63

In previous reports (9, 10) experiments were described which indicated that transplantation of isologous lymphoid cells from immune animals to Lymphotic Choriomeningitis (LCM) virus carrier mice conferred a responsiveness to the virus in the recipients This conferred responsiveness caused a marked decrease in the virus titres in the blood and organs of the virus carrier mice However, with the strain of mice and the cell doses used, total elimination of the virus was not achieved When the virus in the blood was reduced about 10^3-fold and even more of the virus in the spleen had disappeared, no further virus reduction occurred The virus titres stabilized at a very low level and were found to be constant during observation periods of up to three months It is the purpose of this article to present further studies concerning the duration of the transplantation effect on the virus and the possibility of complete elimination of all detectable virus by this procedure

MATERIAL AND METHODS

The LCM virus used was the same as described in the previous papers

The mice used for the transplantation experiments were highly inbred strains of AKA and C3H mice The AKA mice were from the same stock as those described in the foregoing report The C3H mice have been bred at this Institute for several years

The blood for virus titrations was taken from the tail vein the veneous plexus at the canthus of the eye or from the heart In all cases just enough heparin was added to prevent clotting

The virus titrations were carried out by intracerebral inoculation into the Institute's stock of ordinary white Swiss mice (10) The titration endpoints were calculated according to Karber The values presented in the ...

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the us ... counts in the AKA mice have been described previously The effect of the virus inoculations in C3H babies followed the same pattern as the one seen in the AKA mice Only relatively few of the babies died and after a couple of weeks all the rest developed normally and remained virus carriers for the rest of their lives

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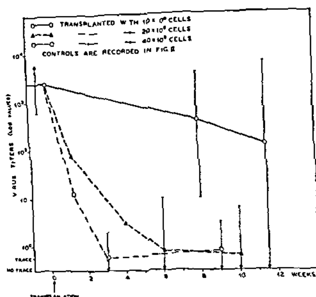


Fig 1

Mean blood virus titre curves for groups of C_3H virus carriers transplanted with 10×10^6, 20×10^6 and 40×10^6 immune lymphoid cells

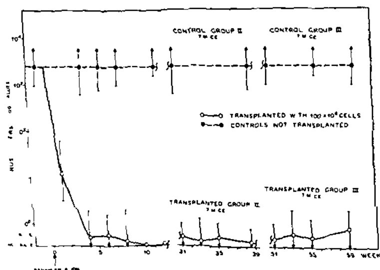


Fig 2

Mean blood virus titre curves for groups of C_3H virus carrier mice transplanted with 100×10^6 immune lymphoid cells ↑ or ↓ indicates that the titration endpoints have not been reached for some mice in the group

Virus titres of the blood of untreated mature virus carriers The virus titres found in the AKA mice corresponded closely to those seen in the foregoing experiments and the mean titre was 10^4 In C_3H mice, however, the virus titre level was somewhat lower In titrations of 145 C_3H virus carrier mice the mean titre was $10^{3.4}$, and the variation from this titre roughly $\pm 10^1$ The lowest titre seen in the 145 untreated C_3H virus carriers was $10^{2.4}$ and only two of the mice had titres lower than $10^{2.5}$

The preparation of lymphoid cells for transplantation and the general experimental conditions followed closely those outlined in the foregoing report

Transplantation route As (10) the intraperitoneal route was found to be just as effective as the intravenous, all transplantations were carried out by intraperitoneal inoculations

EXPERIMENTAL

Duration of the Transplantation Effect of Immune Lymphoid Cells

In the previous experiments with AKA mice, the longest observation time of virus carrier mice after a transplantation was three months Because of the high incidence of spontaneous leukæmia in AKA mice, this strain is not suitable for more protracted experiments Therefore, for longer observation times a more stable mouse strain had to be used and C_3H mice were chosen for the purpose Unfortunately nothing was known about the LCM virus carrier state in these mice and nothing about the effect of transplantations of immune lymphoid cells It was soon found, however, that the virus carrier state could be achieved just as easily in C_3H mice as in AKA, but as described in "Material and Methods", the virus titres are a little lower in C_3H virus carriers than in AKA mice

The effect of adoptive immunization of C_3H virus carriers was investigated in pilot experiments Five groups of six virus carriers were selected One group served as controls and received no treatment, the second group was transplanted with 10×10^6 lymphoid cells, the third with 20×10^6 cells, the fourth with 40×10^6 cells and the fifth with 100×10^6 cells All transplantations were carried out with cells from the same cell pool harvested from donors vaccinated several times The results are recorded in Fig 1 and the left half of Fig 2 It is apparent that when only 10×10^6 cells are transplanted some mice will show a marked decrease in blood virus titres In others, only a weak response is seen, but some mice do not react at all However, when the transplantation dose is raised to 20×10^6 cells or more, all the transplanted mice will respond to the transplantation with a marked decrease of the blood virus titres, and the final result is the same for all groups, *i e*, a stabilized low virus titre level of about 10° How steep the virus reduction curve will be depends on the amount of cells given Transplantation of 100×10^6 lymphoid cells causes a much quicker response than the 20×10^6 cell doses When compared with the transplantation results in AKA virus carriers described in the previous report (10), it will be seen that the C_3H mice react to the transplantation as readily and in the same manner as the AKA mice However in C_3H mice a good result in all transplanted mice can be obtained with two to three times fewer

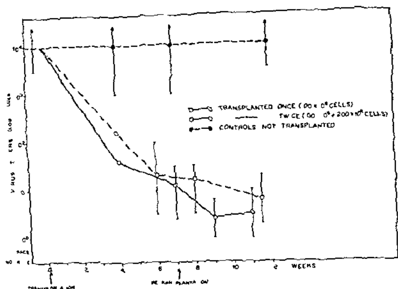


Fig 3
Effect of retransplantation on AKA virus carriers

plantation was very modest Compared with the group which received only one transplantation, only a slight depression of the mean titre curve occurred and in the following six weeks the two mean titre curves followed one another so closely that the difference between them could not be considered significant

The second experiment was carried out exactly as the first, except that the cell dose for the second transplantation was raised to 200 × 10⁶ lymphoid cells However, as recorded in Fig 3, the results obtained were similar to those in the first experiment and in none of the transplanted mice did the blood titre fall below the detectable level

Effect of very high transplantation doses to AKA virus carriers Even if repeated transplantations and cell doses of a total of 300 × 10⁶ immune cells did not cause a greater virus titre reduction than actually could be obtained with a much smaller dose, the possibility still remained that larger doses might change the picture Experiments were therefore carried out with the purpose of investigating that possibility

For the first experiment three groups of AKA virus carriers were selected One received no transplantation and was kept as a control Of the two remaining groups, mice in the first were transplanted with 100 × 10⁶ lymphoid cells per mouse and the second with 1000 × 10⁶ cells The cells used were taken from the same cell pool and originated from donors vaccinated repeatedly The result is shown in Fig 4 It can be seen that the group of m

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cells, and the final blood virus titre level is roughly one log lower than that usually obtained in AKA mice

As indicated in Fig 2, the observation time for the first C_3H mouse transplantation experiment was not more than 12 weeks However, in the course of about a year, two groups of seven transplanted C_3H virus carriers and two control groups were observed for a much longer period Each mouse in the transplanted groups was transplanted with 100×10^6 lymphoid cells from donors vaccinated several times The controls received no transplantation Before the beginning of the experiments the blood from each individual mouse in all the groups was titrated for virus In transplanted group II and control group II the blood virus titre for each individual mouse was determined several times from the 31st to the 39th week, and in transplanted group III and control group III from the 51st to the 59th week after the beginning of the experiment The results are recorded in the right half of Fig 2 It can be seen clearly that in all the mice in the transplanted groups the blood virus titres, brought down by the transplantations, remain at the same level throughout these very long observation periods

In the experiment described above, each individual mouse has been titrated several times during observation periods of eight weeks Therefore data concerning the individual titre variations has also been obtained As from the time when the virus titres had been brought down by the transplantation, virus was never demonstrable in the blood in as many as 11 out of 21 transplanted mice About 25 per cent showed titres which changed from absence of trace to presence of trace, and the rest had always some detectable virus in their blood However, the highest titre measured was only $10^{0.3}$ None of the mice in the control groups had blood virus titres which could be considered to be reduced significantly and the lowest titre observed was $10^{2.8}$

Attempts to Eliminate All Detectable Virus in Virus Carriers

Since sufficient numbers of C_3H mice were not available, the main experiments were carried out on AKA mice

Effect of repeated transplantation of AKA virus carriers In the first experiment, three groups of AKA virus carriers were selected One group received no treatment and acted as a control The two other groups were transplanted with immune cells from donors which had been vaccinated several times All the mice in both these groups received 100×10^6 cells from the same cell pool The blood virus titres of the transplanted mice went down as usual, reaching the stabilized low titre level of about 10^1 in six weeks At that time the mice in one of the groups were transplanted a second time Each individual mouse in this group received 100×10 lymphoid cells from donors vaccinated several times The effect on the virus titres in the mice given this second trans-

were killed and the blood and organs titrated for virus The results are seen in Table 1 It is apparent that there was no trace of virus left in the blood of the transplanted mice and the same was the case as regards the spleen and lymph nodes However, in the lungs, liver, brain and thymus some virus but only a small quantity, could be found Concerning the kidneys it is very striking that this organ in all mice was found to have a rather high virus titre much higher than titres found anywhere else in the transplanted mice

TABLE 1

Virus Content of the Organs of Virus Carrier Mice 8 Weeks after Transplantation of 500 2000 × 10 Immune Lymphoid Cells

		Virus titres (log values)							
		Blood*	Spleen §	Lymph Nodes†	Thymus §	Lungs§	Kidneys§	Liver§	Brain §
AKA mice transpl with 2000 × 10⁶ cells	no 1	n tr	n tr	n tr	2 0	≧2 3	≧2 5	tr	tr
	no 2	n tr	n tr	n tr	n tr	1 5	≧2 5	n tr	1 3
	no 3	n tr	n tr	n tr	≧2 5	2 0	≧2 5	n tr	n tr
	no 4	n tr	n tr	n tr	1 5	1 5	≧2 5	n tr	n tr
C3H mice transpl with 500 × 10⁶ cells	no 1	tr	n tr	n tr	1 0	n tr	3 3	n tr	tr
	no 2	n tr	n tr	n tr	1 0	n tr	2 5	n tr	n tr
	no 3	n tr	n tr	n tr	tr	n tr	2.3	n tr	n tr
	no 4	n tr	n tr	n tr	tr	tr	2 0	n tr	n tr
	no 5	n tr	n tr	n tr	1 3	n tr	3 0	tr	n tr
Control AKA mice n t transpl	no 1	3 3	≧8 5	7 8	6 5	7 3	≧8 5	6 5	4 5
	no 2	3 8	≧8 5	8 0	≧8 5	7 0	≧8 5	6 8	5 8
	no 3	3 8	≧8 5	≧8 5	7.8	7.3	≧8 5	8 0	6 0
	no 4	3 9	≧8 5	≧8 5	≧8 5	7 0	≧8 5	7.8	7 0
	no 5	3 8	≧8 5	≧8 5	8 0	≧8 5	≧8 5	7 0	6 8
Control C3H mice n t transpl	no 1	4 0	7 5	6 8	6 0	5 5	6 5	5 8	6 5
	no 2	3 5	6 5	6 0	5 8	5.3	7 3	6 5	6 5
	no 3	3 5	7 0	6 8	6 0	5 8	6 5	5 0	6 8
	no 4	3 5	6 0	7.3	6 0	5 8	7 0	7 0	6 8
	no 5	3 0	6 8	7 0	5 3	6 0	7 5	7 0	7 5

* = undiluted blood § = 20 per cent supensions † = 10 per cent suspensions
n tr = no trace tr = trace

Titrations of the organs of the control group of untreated virus carriers killed at the same time revealed extremely high virus titres of about 10^8 in the spleen lymph nodes thymus and kidneys and more moderate titres in the rest of the organs As the spleen and lymph nodes are among the organs with the highest titres in the untreated virus carriers and these organs are the only ones completely free of virus in the transplanted group it is clear that it is not the original amount of virus in a given place which determines the effect of an adoptive immunization

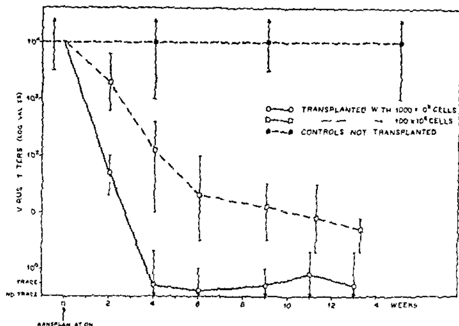


Fig 4

Effect of very high transplantation doses on AkA virus carriers

Moreover, already after three weeks, four out of the six mice in this group had reached a virus titre level so low that no virus could be detected in undiluted blood However, this great effect on the virus was only temporary In the course of the following weeks the virus began to reappear in the blood, and when each mouse was observed individually it was found that in all the mice the blood virus titre undulated up above and down below the detectable level throughout the whole observation period of 13 weeks

A new attempt was made, using even higher cell doses The first part of this experiment was exactly the same as the one just described One

and one group of 4 mice were transplanted with 1000 × 10^6 lymphoid cells per mouse The lymphoid cells used were taken from the same cell pool and as usual harvested from donors vaccinated repeatedly Three weeks later the group in which each mouse had received 1000 × 10^6 cells were retransplanted with 1000 × 10^6 lymphoid cells per mouse The cells used for this second transplantation were likewise from donors vaccinated repeatedly The results showed the ordinary virus titre curve for the group of mice receiving 100 × 10^6 lymphoid cells reaching a stabilized low titre level of about 10^1 in five weeks However, in the mice transplanted twice with 1000 × 10^6 lymphoid cells no trace of virus was found in undiluted blood in any of the mice two weeks after the second transplantation Five weeks after the second transplantation, i e , eight weeks after the beginning of the experiment, all four mice

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AKA mice transpl with 2000 × 10^6 cells	no 1	n tr	n tr	n tr	2.0	≥2.3	≥2.5	tr	tr
	no 2	n tr	n tr	n tr	n tr	1.5	≥2.5	n tr	1.3
	no 3	n tr	n tr	n tr	≥2.5	2.0	≥2.5	n tr	n tr
	no 4	n tr	n tr	n tr	1.5	1.5	≥2.5	n tr	n tr
C_3H mice transpl with 500 × 10^6 cells	no 1	tr	n tr	n tr	1.0	n tr	3.3	n tr	tr
	no 2	n tr	n tr	n tr	1.0	n tr	2.5	n tr	n tr
	no 3	n tr	n tr	n tr	tr	n tr	2.3	n tr	n tr
	no 4	n tr	n tr	n tr	tr	tr	2.0	n tr	n tr
	no 5	n tr	n tr	n tr	1.3	n tr	3.0	tr	n tr
Control AKA mice not transpl	no 1	3.3	≥8.5	7.8	6.5	7.3	≥8.5	6.5	4.5
	no 2	3.8	≥8.5	8.0	≥8.5	7.0	≥8.5	6.8	5.8
	no 3	3.8	≥8.5	≥8.5	7.8	7.3	≥8.5	8.0	6.0
	no 4	3.9	≥8.5	≥8.5	≥8.5	7.0	≥8.5	7.8	7.0
	no 5	3.8	≥8.5	≥8.5	8.0	≥8.5	≥8.5	7.0	6.8
Control C_3H mice not transpl	no 1	4.0	7.5	6.8	6.0	5.5	6.5	5.8	6.5
	no 2	3.5	6.5	6.0	5.8	5.3	7.3	6.5	6.5
	no 3	3.5	7.0	6.8	6.0	5.8	6.5	5.0	6.8
	no 4	3.5	6.0	7.3	6.0	5.8	7.0	7.0	6.8
	no 5	3.0	6.8	7.0	5.3	6.0	7.5	7.0	7.5

* = undiluted blood § = 20 per cent supensions † = 10 per cent suspensions
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Titrations of the organs of the control group of untreated virus carriers killed at the same time revealed extremely high virus titres of about 10^8 in the spleen lymph nodes, thymus and kidneys and more moderate titres in the rest of the organs As the spleen and lymph nodes are among the organs with the highest titres in the untreated virus carriers and these organs are the only ones completely free of virus in the transplanted group, it is clear that it is not the original amount of virus in a given place which determines the effect of an adoptive immunization

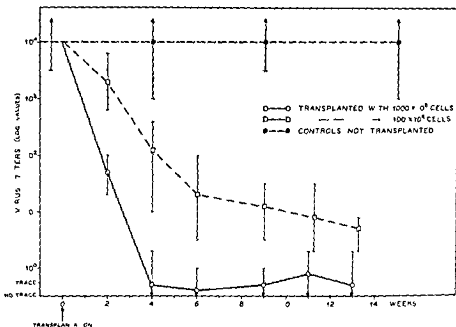


Fig 4
Effect of very high transplantation doses on AkA virus carriers

Moreover, already after three weeks, four out of the six mice in this group had reached a virus titre level so low that no virus could be detected in undiluted blood However, this great effect on the virus was only temporary In the course of the following weeks the virus began to reappear in the blood, and when each mouse was observed individually it was found that in all the mice the blood virus titre undulated up above and down below the detectable level throughout the whole observation period of 13 weeks

A new attempt was made, using even higher cell doses The first part of this experiment was exactly the same as the one just described One group of five virus carriers received no treatment and served as controls, one group of five mice received 100×10^6 lymphoid cells per mouse, and one group of 4 mice were transplanted with 1000×10^6 lymphoid cells per mouse The lymphoid cells used were taken from the same cell pool and as usual harvested from donors vaccinated repeatedly Three weeks later the group in which each mouse had received 1000×10^6 cells were retransplanted with 1000×10^6 lymphoid cells per mouse The cells used for this second transplantation were likewise from donors vaccinated repeatedly The results showed the ordinary virus titre curve for the group of mice receiving 100×10^6 lymphoid cells reaching a stabilized low titre level of about 10^1 in five weeks However, in the mice transplanted twice with 1000×10^6 lymphoid cells no trace of virus was found in undiluted blood in any of the mice two weeks after the second transplantation Five weeks after the second transplantation, *i e*, eight weeks after the beginning of the experiment all four mice

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	no 2	n tr	n tr	n tr	n tr	1 5	≧2 5	n tr	1.3
	no 3	n tr	n tr	n tr	≧2 5	2 0	≧2 5	n tr	n tr
	no 4	n tr	n tr	n tr	1 5	1 5	≧2 5	n tr	n tr
C_3H mice transpl with 500 × 10^6 cells	no 1	tr	n tr	n tr	1 0	n tr	3 3	n tr	tr
	no 2	n tr	n tr	n tr	1 0	n tr	2 5	n tr	n tr
	no 3	n tr	n tr	n tr	tr	n tr	2 3	n tr	n tr
	no 4	n tr	n tr	n tr	tr	tr	2 0	n tr	n tr
	no 5	n tr	n tr	n tr	1 3	n tr	3 0	tr	n tr
Control AKA mice n t transpl	no 1	3 3	≧8 5	7 8	6 5	7 3	≧8 5	6 5	4 5
	no 2	3 8	≧8 5	8 0	≧8 5	7 0	≧8 5	6 8	5 8
	no 3	3 8	≧8 5	≧8 5	7 8	7.3	≧8 5	8 0	6 0
	no 4	3 9	≧8 5	≧8 5	≧8 5	7 0	≧8 5	7.8	7 0
	no 5	3 8	≧8 5	≧8 5	8 0	≧8 5	≧8 5	7 0	6 8
Control C_3H mice n t transpl	no 1	4 0	7 5	6 8	6 0	5 5	6 5	5 8	6 5
	no 2	3 5	6 5	6 0	5 8	5 3	7 3	6 5	6 5
	no 3	3 5	7 0	6 8	6 0	5 8	6 5	5 0	6 8
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Effect of high transplantation doses to C3H virus carriers As described above, the minimum dose of immune lymphoid cells required to cause a good transplantation effect on the virus in all C3H recipients is about 20 × 10^6 cells As the immune cell dose required for a good transplantation effect in AKA mice is about 50 × 10^6 immune lymphoid cells (10), it seems reasonable to assume that the effect of 500 × 10 cells to C3H mice would be about equal to 2000 × 10^6 cells in AKA mice For this reason the transplantation effect of 500 × 10^6 immune lymphoid cells in C3H virus carriers was investigated Each of five C3H virus carriers was transplanted with this cell dose, five were transplanted with 100 × 10^6 cells per mouse (from the same cell batch), and five received no transplantation The cells used for transplantation were harvested from highly immune donors (= vaccinated mothers to infected babies, see (10)) The effect of the 100 × 10^6 cells was closely similar to that obtained in the experiment recorded in Fig 2 However, in the group which received 500 × 10^6 cells per mouse, all detectable virus had disappeared from the blood of all mice in the course of three weeks and the virus never reappeared during the following observation period of five weeks At that time, *i e* eight weeks after the beginning of the experiment, all mice transplanted with 500 × 10^6 cells and all the untreated controls were killed and the blood and organs titrated for virus The results are recorded in Table 1 As was the case with AKA virus carriers transplanted with 2000 × 10^6 immune lymphoid cells, none of the transplanted C3H virus carriers were completely free of virus Moreover, it was again the kidneys which had the highest titres and the spleen and lymph nodes which had none In one mouse a trace of virus was found even in the blood, but that does not seem surprising in view of the rather high virus titres in some other organs The organs of the control mice had, on the whole, lower virus titres than those seen in the AKA mice However, also in C3H mice the spleen and lymph nodes were among the organs where the highest virus titres were found

DISCUSSION

The experiments described here have shown that a virus carrier state can be established as easily in C3H mice as in AKA mice Moreover, the reduction of the virus in virus carriers caused by transplantation of immune lymphoid cells follows the same pattern in both these mouse strains Other mice have not yet been tested but as AKA and C3H mice genetically are rather different it seems reasonable to assume that, except for minor variations, many more mouse strains can be found which will behave in the same manner

In the virus carrier mice there seems to be a certain balance between the virus and the host, a balance which causes extremely high virus titres in many organs, especially in the spleen lymph nodes thymus and kidneys If left alone, only minor changes in this balance seem to

occur throughout the whole life of the mouse Thus we have groups of C3H virus carriers observed for one year and a half showing the same blood virus titres as found shortly after they were infected neonatally Furthermore, in other groups kept nearly as long, the virus titres of the organs were similar to those seen in very young mice (*Hannover Larsen* (2)) However, when the virus carriers are transplanted with a sufficiently high dose of immune lymphoid cells, then the balance changes dramatically in favour of the host As a result, a very low virus titre level in the blood and organs is established The new balance causing this change is also permanent, showing no sign of alteration during observation times of up to more than a year

The cell dose necessary for causing an alteration of the virus-host balance depends on the cells, the strain of mice used and the individual recipient Concerning the cells, it was shown in the previous report (10) that the vaccination status of the donor plays only a minor rôle However in our study, the best and most uniform results have been obtained with cells harvested from mothers to infected babies (These donors had been immunized by the natural infection acquired from the babies and later on by an experimental virus challenge) The strain influence is apparent from the fact that a good response can be achieved in C3H mice with two to four times fewer cells than in AKA mice Moreover, the final virus titre level obtained in C3H mice is about one log lower in C3H than in AKA mice This last difference, however, may not be caused by a difference in transplantation response but might be due to the lower virus titre level usually found in untreated C3H virus carrier mice The influence of the host factor is most clearly seen when small cell doses are transplanted Some mice will then show a quick and marked decrease in the blood virus titres, others will show a slow response, and some will not react at all When the cell dose is increased to what can be called an "effective" dose then all mice will respond to the transplantation with a decrease of the virus titres The time factor for the response to this dose may vary, but the final result will be similar for all, i e , a reduction of the blood virus titres of about 99 9 per cent and an even greater titre reduction in organs such as the spleen and lymph nodes When the effective transplantation dose is raised five to ten times the individual variations are reduced but the final titre level will be the same Still some virus will be present in the blood Not even *repeated transplantations* with high cell doses can clear the blood of the last tiny amount of virus Extremely high cell doses—doses which are about 40 times higher than the "effective dose" and which correspond to about all the spleen and lymph node cells which can be obtained from ten donor mice—can usually remove all detectable virus in the blood However, not even these huge transplantation doses can clear the mice completely Some virus has always been found somewhere All these results indicate strongly that the effect of the smallest transplantation doses which can cause a virus titre reduction in all

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The experiments described here have shown that a virus carrier state can be established as easily in C_3H mice as in AKA mice Moreover, the reduction of the virus in virus carriers caused by transplantation of immune lymphoid cells follows the same pattern in both these mouse strains Other mice have not yet been tested, but as AKA and C_3H mice genetically are rather different it seems reasonable to assume that, except for minor variations, many more mouse strains can be found which will behave in the same manner

In the virus carrier mice there seems to be a certain balance between the virus and the host, a balance which causes extremely high virus titres in many organs, especially in the spleen, lymph nodes thymus and kidneys If left alone, only minor changes in this balance seem to

occur throughout the whole life of the mouse Thus we have groups of C3H virus carriers observed for one year and a half showing the same blood virus titres as found shortly after they were infected neonatally Furthermore, in other groups kept nearly as long, the virus titres of the organs were similar to those seen in very young mice (*Hannover Larsen* (2)) However, when the virus carriers are transplanted with a sufficiently high dose of immune lymphoid cells, then the balance changes dramatically in favour of the host As a result, a very low virus titre level in the blood and organs is established The new balance causing this change is also permanent, showing no sign of alteration during observation times of up to more than a year

The cell dose necessary for causing an alteration of the virus host balance depends on the cells, the strain of mice used and the individual recipient Concerning the cells, it was shown in the previous report (10) that the vaccination status of the donor plays only a minor rôle However, in our study, the best and most uniform results have been obtained with cells harvested from mothers to infected babies (These donors had been immunized by the natural infection acquired from the babies and later on by an experimental virus challenge) The strain influence is apparent from the fact that a good response can be achieved in C3H mice with two to four times fewer cells than in AKA mice Moreover, the final virus titre level obtained in C3H mice is about one log lower in C3H than in AKA mice This last difference, however, may not be caused by a difference in transplantation response but might be due to the lower virus titre level usually found in untreated C3H virus carrier mice The influence of the host factor is most clearly seen when small cell doses are transplanted Some mice will then show a quick and marked decrease in the blood virus titres, others will show a slow response, and some will not react at all When the cell dose is increased to what can be called an 'effective' dose, then all mice will respond to the transplantation with a decrease of the virus titres The time factor for the response to this dose may vary, but the final result will be similar for all *i e* a reduction of the blood virus titres of about 99 9 per cent and an even greater titre reduction in organs such as the spleen and lymph nodes When the effective transplantation dose is raised five to ten times the individual variations are reduced but the final titre level will be the same Still some virus will be present in the blood Not even repeated transplantations with high cell doses can clear the blood of the last tiny amount of virus Extremely high cell doses—doses which are about 40 times higher than the "effective dose" and which correspond to about all the spleen and lymph node cells which can be obtained from ten donor mice—can usually remove all detectable virus in the blood However not even these huge transplantation doses can clear the mice completely Some virus has always been found somewhere All these results indicate strongly that the effect of the smallest transplantation doses which can cause a virus titre reduction in all

transplanted virus carriers is already very close to the maximum effect which can be obtained. Therefore, the virus reduction in virus carriers induced by transplantation seems to be almost an all-or-nothing phenomenon.

Already 30 years ago, *Traub* (6, 7) found that although adult mice infected artificially with LCM virus have a natural tendency to free themselves of the virus, a complete clearance was not always achieved. Under certain conditions, a rather protracted carrier state could be created. Other investigators (3, 5, 6) have confirmed this observation, and also we have detected small amounts of virus in immunized mice a long time after the vaccination had been performed (11). It is not too surprising, therefore, that even the highest transplantation doses given in our experiments have failed to clear completely the virus carriers of their virus. However, this problem will be discussed further in a subsequent report. The fact that some virus remain in the transplanted virus carriers indicates the persistence of virus producing cell foci. These cells seem to be localized in many organs, especially in the kidneys. However, it is very striking that the spleen and lymph nodes are the only organs which can be freed of any detectable virus by transplantation of immune lymphoid cells, notwithstanding that the spleen and the lymph nodes belong to the organs which in untreated virus carriers have the highest virus titres—much higher than the lungs or the liver, which often are not cleared by the transplantation. The phenomenon might perhaps be due to the fact that transplanted spleen and lymph node cells mainly settle in the spleen and lymph nodes of the recipient, whereas only few cells go to other organs (4). However, one wonders if this is the most likely explanation as regards the kidneys. Kidneys seem to be organs which particularly attract viruses. Many viruses grow well or best in kidney tissue cultures and quite a few have been found hidden in kidney cells. The reason for this is completely unknown. However, the fact that kidney tissue often has a high anti-complementary activity (1) might perhaps play a rôle.

SUMMARY

The virus carrier state is as easily established in C_3H mice, as in AKA mice.

The C_3H virus carriers respond to adoptive immunization in the same manner as AKA mice.

The stabilized low virus titre level caused by a transplantation of lymphoid cells from immune donors is permanent.

The transplantation effect on the virus in virus carriers is close to an all-or-nothing phenomenon.

Very high transplantation doses of immune lymphoid cells clear the spleen and lymph nodes of all detectable virus. However, a complete

clearance of a mouse has not been achieved Even after the highest transplantation dose given some virus has been found somewhere, especially in the kidneys

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tribute to the confusion, either by the way in which the description is formulated or by the obvious discrepancies existing between different authors Thus, *P aeruginosa* is usually described simply as having "1-3 flagella at one pole", a formulation which is open to a number of interpretations The statements about *P fluorescens* vary according to *Bergey's* Manual (1957) and *Prevot*'s handbook (1961) there is only one polar flagellum, *Topley & Wilson* (1955) says there is "one or more polar flagella", and *Krassilnikov* (1959) that gelatin liquefying *P fluorescencs* has 3 to 4, and non-liquefying strains have 1 to 2 flagella

Krassilnikov, in his book (1959) shows a picture of *Pseudomonas pyocyanea* with "polare lophotriche Geisseln (2 oder mehr)", and in *Leifson's* Atlas (1960) the only picture of a named *P fluorescens* strain shows a cell with one polar flagellum If these recent pictures were accepted as representative, one would have a situation directly opposite to the one shown in *Migula*'s old pictures

It is perhaps understandable if in this situation a majority of bacteriologists have *preferred to follow* those who discourage the use of flagella number in identification and taxonomy of pseudomonads However, experience through several years in this laboratory had given the impression that the facts are not nearly as confusing as the literature indicates and therefore it was decided to make a systematic examination of the number of flagella in a large number of strains in an attempt to clarify the situation

MATERIAL AND METHODS

Strains

[illegible]

[illegible] selected tests

The flagella study [illegible]
the largest number [illegible]
are shown in Table [illegible]
strains in this biotype [illegible] and the remaining ones conformed in all other respects to the pyocyanin forming ones Biotype B seems to correspond to *P ovalis* or *P fluorescens nonliquefaciens* and biotypes C and D to *P fluorescens liquefaciens* divided into two varieties differing in the [illegible]

[illegible] partly because of the special interest pertaining to the difference in flagellation between *P aeruginosa* on the one hand and the other commonly occurring green fluorescent pseudomonads on the other hand

Staining Technique

A flagella stained preparation was made of each of the 832 strains using *Leifson's* staining technique (*Leifson* 1960) The basic fuchsin was a mixture of 3 parts of pararosanilinhydrochloride and 1 part of pararosanilinacetate obtained from Edw Gurr London

FROM THE DEPARTMENT FOR DIAGNOSTIC BACTERIOLOGY, STATENS SERUM-INSTITUT, COPENHAGEN, DENMARK (HEAD HANS LAUTROP, M D)

ON THE DISTINCTION BETWEEN POLAR MONOTRICHOUS AND LOPHOTRICHOUS FLAGELLATION IN GREEN FLUORESCENT PSEUDOMONADS

By

HANS LAUTROP and OVE JESSEN

Received 3 x 63

All green fluorescent pseudomonads—if motile—have polar flagella The number of flagella may be one or several and the idea early arose that the number might be correlated with other characteristics and therefore a character of taxonomic value

Fischer (1894, 1897) and *Migula* (1897–1900) were the first to consider this possibility Using *Messea's* terms mono- and lophotrichous they distinguish between organisms with a single polar flagellum and others having a tuft of flagella at the pole *Fischer* used the distinction at the generic level in his taxonomic system, but *Migula* whose system won more general acceptance combined all straight rods with polar flagella—irrespective of the number—in the single genus *Pseudomonas* He nevertheless admitted that some species in the genus had a characteristic flagellation, *e g P aeruginosa* is described as having "stets nur eine polare Geissel" although it was recognized that a few cells with two or even three polar flagella might be encountered, and of *P fluorescens* (Flugge) *Migula* says that "sie besitzen polare Geisselbuschel" Quite good photomicrographs illustrate *Migula's* statements concerning the flagellation of these two species

The medically important distinction between *P aeruginosa* and [illegible] *lla* described [illegible] ation is more [illegible] within later years the situation has become rather confused In 1949 *Bartholomew* reached the conclusion that, due to variations in the number of flagella, both among strains from a single species and among cells from an individual culture, it was impossible to distinguish between monotrichous and lophotrichous flagellation *Rhodes* (1958) obtaining similar results, proposed to abandon completely the use of flagella number in *Pseudomonas* taxonomy and her view was later adopted by *Buttiaux & Gagnon* (1958/59), *Lysenko* (1961) and *Colwell & Liston* (1961)

The statements concerning flagellation in modern handbooks con-

tribute to the confusion, either by the way in which the description is formulated or by the obvious discrepancies existing between different authors. Thus, *P. aeruginosa* is usually described simply as having '1–3 flagella at one pole', a formulation which is open to a number of interpretations. The statements about *P. fluorescens* vary according to *Bergey's* Manual (1957) and *Prevot's* handbook (1961) there is only one polar flagellum. *Topley & Wilson* (1955) say there is 'one or more polar flagella', and *Krassilnikov* (1959) that gelatin liquefying *P. fluorescencs* has 3 to 4 and non liquefying strains have 1 to 2 flagella.

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It is perhaps understandable if in this situation a majority of bacteriologists have preferred to follow those who discourage the use of flagella number in identification and taxonomy of pseudomonads. However experience through several years in this laboratory had given the impression that the facts are not nearly as confusing as the literature indicates and therefore it was decided to make a systematic examination of the number of flagella in a large number of strains in an attempt to clarify the situation.

MATERIAL AND METHODS

Strains

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man [illegible]
was [illegible]
el ex [illegible]
divi [illegible]
iden [illegible]

The [illegible]
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are [illegible]
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The [illegible] four largest and most commonly occurring biotypes were chosen for study partly because a relatively large number of supposedly [illegible]

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TABLE 1

Selected Cultural and Biochemical Characteristics of 4 Biotypes of Green Fluorescent Pseudomonads

Bio type	Name used for biotype	Number of strains	Growth at 37°C	Gelatin liquefaction	Reduction of NO_3^- to NO_2^-	Reduction of NO_2^- to N_2	Acid from: Sucrose	Trehalose	Mannitol	Adonitol & Inositol	Haemolysis	Egg yolk reaction
A	P. aeruginosa	334	+	+	+	+	—	d	+	—	+	—
B	P. ovalis	93	d	—	—	—	—	—	—	—	—	—
C	P fluorescens	72	—	+	+	+	—	+	+	+	+	+
D	P. fluorescens	44	d	+	—	—	+	+	+	+	+	+

acidification

The bacteria were usually suspended in distilled water without preliminary washing from an overnight culture grown on infusion broth agar at room temperature or at 30° C If a satisfactory preparation could not be obtained in this way, a washed, formol treated cell suspension from a fluid culture at room temperature or 30° C was used to make a new preparation The fluid medium contained Bacto Tryptone Difco 0 5 per cent, yeast extract Difco 0 1 per cent mono- and dibasic potassiumphosphate 0 05 per cent of each Fluid cultures in the same medium, but grown at 10° C, were used when a strain was re examined to study the effect of low temperature on the number of flagella

Evaluation of the Stained Preparations and Counting of Flagella Numbers

classified as mono- or lophotrichous according to *one, respectively several, polar flagella* Fixed cr 'wo classes were not set up beforehand, and in about 100 strains a question-mark was used to indicate the uncertainty of the decision

Next an actual counting of flagella was performed on strains of the four chosen biotypes using the already existing preparations For technical reasons it was necessary to use only the best suited preparations for counting *i e* slides having areas with many but well dispersed cells and perfectly stained flagella Therefore the number of strains actually counted is smaller than the number of strains recorded in Table 1

As a third stage in the study a flagella counting was performed in the nearly 100 strains outside the chosen biotypes which had received a question mark at the first evaluation as mono- or lophotrichous The counting of these strains was performed from fluid pa cu e number of flagella on the ... mounted with a ruled eyepiece plate to facilitate the counting To include a cell in the counting it was required that it should be possible to determine the number of ... cells d each f cells were excluded because it was assumed that they represent cells in an early stage of division In the counting the number of flagella were scored as 1 2 3 and more than 3 flagella per pole. It was found that when more than 3 flagella were present it was too often impossible to decide exactly how many there were

RESULTS

1 Flagella Counting in Selected Groups of Green Fluorescent Pseudomonads and Definition of the Flagellar Index

The results of the counting are summarized in Tables 2 and 3 From the figures in Table 2 it can be seen that there is a striking difference between *P aeruginosa* on the one side and the biotypes B C and D on the other side It was found that the simplest way to express the difference was to indicate the number of cells having more than one flagellum —irrespective of the actual number—as a percentage of all flagellated cells This figure is for the sake of convenience called the flagellar index a low index thus indicates that there are only few cells with more than one flagellum and a high index that there are many

TABLE 2

Result of Flagella Counting in 4 Different Biotypes of Green Fluorescent Pseudomonads

Bio-type	Name used for biotype	Number of strains	Total number of cells counted	Percentage of flagellated cells having the indicated number of flagella				Flagella index
				1	2	3	>3	
A	P aeruginosa	122	6025	97	3	0 05	0	3
B	P ovalis	40	2250	33	33	21	13	67
C	P fluorescens	35	3400	48	29	13	10	52
D	P fluorescens	28	1590	50	33	13	4	50

The number of flagella was counted on 50 or 100 individual cells from each strain in a Leifson stained preparation The flagella index is the combined percentages of all flagellated cells having more than one flagellum per pole

In Table 3 the individual strains have been arranged according to the numerical value of their indices and it can be seen that all strains of *P aeruginosa*—with one exception—have indices equal to or below 10 whereas all the other strains—with two exceptions—have indices equal to or above 25

In this selected part of the strain material it thus seemed possible to distinguish clearly between two morphological groups of green fluorescent pseudomonads and this division was correlated with a division based on cultural and biochemical characteristics

It was therefore decided to distinguish tentatively between a low index group and high index group separated by a border area comprising strains with index values between 11 and 24 and to examine how the remaining strains of the material would be distributed on these two morphological groups

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C	P fluorescens	72	—	+	+	+	—	+	+	+	+	+
D	P fluorescens	44	d	+	—	—	+	+	+	+	+	+

The symbol + indicates growth liquefaction reduction or acidification
The symbol — indicates no growth no liquefaction no reduction or no acidification
The symbol d *indicates that individual strains may be either + or —*

The bacteria were usually suspended in distilled water without preliminary washing from an overnight culture grown on infusion broth agar at room temperature or at 30° C If a satisfactory preparation could not be obtained in this way a washed formol treated cell suspension from a fluid culture at room temperature or 30° C was used to make a new *preparation The fluid medium contained* Bacto Tryptone Difco 0 5 per cent yeast extract Difco 0 1 per cent mono and dibasic potassiumphosphate 0 05 per cent of each Fluid cultures in the same medium but grown at 10° C were used when a strain was re examined to study the effect of low temperature on the number of flagella

Evaluation of the Stained Preparations and Counting of Flagella Numbers

At first all strains were tentatively classified as mono or lophotrichous according to the predominance of cells having one respectively several polar flagella Fixed criteria for the delimitation of the two classes were not set up beforehand and in about 100 strains a question mark was used to indicate the uncertainty of the decision

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slightly higher than 25 were re-examined from fluid cultures grown at 10° C. As a control a number of strains with index values below 10 were re-examined under the same conditions. None of the strains with an index below 10—including 20 strains of *P. aeruginosa*—showed any change as a result of growth at the low temperature, whereas a majority of the strains with intermediate values acquired considerably higher indices under these circumstances. It was decided that the highest index value obtainable was the one that should be used to characterize the strain.

It should be noticed here that at 10° C. it is not always possible with small inocula to obtain sufficiently dense cultures after overnight incubation; this applies particularly to strains of *P. aeruginosa*. Therefore some cultures were not examined until they were 3 to 5 days old. The difference in the age of the cultures thus introduced in the comparison may be of some importance. A few experiments indicated this, but the question has not been thoroughly examined.

By the above mentioned different steps practically all strains were at last classified as to index. The actual values were known for about 300 strains and the remaining ones were classified as having either a low index (i. e. ≦ 10) or a high index (i. e. ≧ 25). The 4 strains in which it proved impossible to obtain a satisfactory staining of the flagella were excluded from the study.

TABLE 4

Distribution of 828 Green Fluorescent Pseudomonads according to Index Group and Biotype

Number of strains	Number of strains in the index groups			Number of biotypes
	Low (≦ 10)	Intermediate 11–24	High (≧ 25)	
9	9			4
334	333	1[b]		1[a]
30		6[c]	24	4
455			455	73
828[d]	342	7	479	82

a Biotype A (*P. aeruginosa*).
b Index value 15.
c Index values 18, 18, 20, 21, 23 and 24.
d Four strains in which flagella staining was not successful are left out.

The result of the correlation of index and biotype in all strains is shown in Table 4. It is evident that there is a positive correlation in the sense that within most of the individual biotypes all strains belong to the same index group. This shows that a definite combination of cultural and biochemical characteristics is regularly accompanied by a definite type of flagellation as expressed by index. The exceptions are few: only 7 strains belonging to 5 different biotypes. Their actual index

TABLE 3
Strains from 4 Different Biotypes of Green Fluorescent Pseudomonads Arranged According to Flagella Index and Diagnosis

Index	A P aeruginosa	B P ovalis	C P fluorescens	D P fluorescens
< 1	47			
1– 2	26			
3– 4	29			
5 6	13			
7– 8	4			
9 10	2			
11 12				
13–14				
15–16	1			
17–18				
19 20				
21–22				
23–24		1	0	1
25 26		0	3	0
27 28		0	1	1
29 30		1	2	0
31–40		0	4	5
41–50		2	5	8
51–60		5	6	7
61 70		11	8	2
71 80		11	5	4
81 90		7	1	0
91–100		2	0	0

The figures in the table indicate number of strains

2 *Application of the Flagellar Index to the whole Collection of 832 Green Fluorescent Pseudomonads*

To avoid the labour involved in a precise determination of the flagellar index in all the remaining strains of the collection, the assumption was made that strains which at the first examination it had been possible to classify either as obviously monotrichous or obviously lophotrichous belonged in the low-index-group or in the high-index-group respectively This assumption seemed justified because it had been regularly confirmed during the examination of the selected groups

The 100 strains which had not given a clear-cut result at the first examination were re examined If possible, a counting was made on the existing slides, otherwise a new preparation was made, this time from a fluid culture

During this part of the study it was noticed that some strains which, according to their cultural and biochemical characteristics, would have been expected to fall into the high-index-group actually had indices lying in or very close to the border line area In studying these strains more closely it was found that if they were grown at 10° C, their index went up considerably Therefore, all strains with an index value between 10 and 25 and a number of strains having index values only

slightly higher than 25 were re-examined from fluid cultures grown at 10° C. As a control a number of strains with index values below 10 were re-examined under the same conditions. None of the strains with an index below 10—including 20 strains of *P. aeruginosa*—showed any change as a result of growth at the low temperature, whereas a majority of the strains with intermediate values acquired considerably higher indices under these circumstances. It was decided that the highest index value obtainable was the one that should be used to characterize the strain.

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By the above mentioned different steps practically all strains were at last classified as to index. The actual values were known for about 300 strains and the remaining ones were classified as having either a low index (*i. e.* $\leqq 10$) or a high index (*i. e.* $\geqq 25$). The 4 strains in which it proved impossible to obtain a satisfactory staining of the flagella were excluded from the study.

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9–10	2			
11–12				
13–14				
15–16	1			
17–18				
19–20				
21–22				
23–24		1	0	1
25–26		0	3	0
27–28		0	1	1
29–30		1	2	0
31–40		0	4	5
41–50		2	5	8
51–60		5	6	7
61–70		11	8	2
71–80		11	5	4
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To avoid the labour involved in a precise determination of the flagellar index in all the remaining strains of the collection, the assumption was made that strains which at the first examination it had been possible to classify either as obviously monotrichous or obviously lophotrichous belonged in the low-index-group or in the high-index-group respectively. This assumption seemed justified because it had been regularly confirmed during the examination of the selected groups.

The 100 strains which had not given a clear cut result at the first examination were re-examined. If possible, a counting was made on the existing slides, otherwise a new preparation was made, this time from a fluid culture.

During this part of the study it was noticed that some strains which, according to their cultural and biochemical characteristics, would have been expected to fall into the high-index-group actually had indices lying in or very close to the border line area. In studying these strains more closely it was found that if they were grown at 10° C, their index went up considerably. Therefore, all strains with an index value between 10 and 25 and a number of strains having index values only

monad makes it very likely that the strain belongs to the high-index-group, if the strain belonged to the low-index-group one would have to search the slide to find them

The high index-group comprises 77 biotypes with altogether 496 strains It has not yet been definitely decided how to combine the biotypes into taxonomic units which deserve specific rank It is clear, however, from what has already been said about the strains that *P fluorescens*—both gelatin liquefying and non-liquefying varieties—belongs to the high-index-group and constitutes a considerable part of it

DISCUSSION

The results of this study have confirmed as well the older observations of *Migula* and *Fischer*, that flagella number is useful in the characterization of green fluorescent pseudomonads, as the more recent ones of *Bartholomew* and *Rhodes*, that the number of flagella is subject to a considerable variation Also, during this study it became obvious how it would be possible to put an end to the confusion which nowadays prevails in these matters

There are probably four main reasons for the prevailing confusion concerning the use of flagella number in *Pseudomonas* taxonomy

1) The poor state of the taxonomy of the group in general, 2) the bad habit to abbreviate flagella descriptions in the literature so much that they become obscure, 3) the variation in the number of flagella both among the individual cells in a culture and among strains belonging to the same species or biotype, 4) the vague definitions of the terms mono-, lopho-, and multitrichous

As long as a satisfactory or recognized subdivision of the group does not exist, the identity of strains studied by various workers will almost always be questionable even if the same names are used In these circumstances, progress in the exact evaluation of the taxonomic importance of the individual characteristics—including flagella number—must of necessity be slow

The use of abbreviated flagellar descriptions is perhaps understandable especially in textbooks and systematic treatises, but they are probably more harmful than imagined For instance, the expression "1–3 polar flagella may—strictly speaking—be true both of low-indexed strains *e g P aeruginosa*, and of high indexed strains, *e g P fluorescens*, but does not give a satisfactory characterization of any of them

The range of the variation in flagella number within the taxonomic entities of the group has never been determined satisfactorily and the various descriptive terms have never been defined exactly Together these two facts constitute the chief reason responsible for the existing confusion

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values are shown in Table 4 One strain is an otherwise typical *P aeruginosa* with the index value 15, and the remaining 6 strains belong to 4 different biotypes in which all other strains belong to the high-index-group, so it may be assumed that they represent extreme variants of this group

It would be possible in the present material to define the two index groups so that the intermediate strains were absorbed However, it cannot be excluded that a limited degree of overlapping between the index groups does occur in nature and later observations of a few strains of *P aeruginosa* with indices between 15 and 21 have confirmed that some caution is advisable in fixing the extreme range of variation acceptable for each of the two groups On the other hand, the agreement between the results in the selected biotypes and in the whole material and the fact that about 99 per cent of the observations fall within the tentatively defined groups indicates that the proposed limits are reasonable In these circumstances it was decided to preserve the proposed groups, *i e* to define a strain having an index value $\leqq 10$ as belonging to the low-index-group and a strain having an index value $\geqq 25$ as belonging to the high index-group and to leave strains with index values between 11 and 24 as not immediately classifiable Such strains will have to be judged on their merits and will require a study of the index value at low temperature and possibly after varying periods of growth

The number of strains which cannot finally be referred to one of the two index groups will probably be very small The possibility that there are strains which inherently have a flagellation which after an index determination would place them in the border area between the indices 11 and 24 has been considered Although the possibility cannot be excluded and even seemed likely before studies at 10° C were performed, the final results are against the likelihood that such biotypes or groups occur

The strains from the 5 biotypes belonging to the low-index-group in the present material are 334 strains of *P aeruginosa*, 4 strains of *P lacrolens* and 5 strains belonging to 3 other biotypes Four of these latter 5 strains are distributed on two very similar biotypes and have so much in common that they should probably be classified together in one taxonomic unit It is surprising that there seems to be only 3 or 4 different species of green fluorescent pseudomonads belonging to the low-index-group Of course, more species may exist but it is not very likely that they are frequently occurring organisms

It is worth noticing from the figures in Table 3 that 93 per cent of the aeruginosa strains have an index $\leqq 6$ It may also be mentioned here that the very low number of cells with 3 flagella in this species was checked by counting the flagella of 1000 cells in each of 4 strains Altogether only two such cells were found, *i e* 0 05 per cent Therefore the observation in rapid succession of even a few cells with 3 polar flagella in a flagella stained preparation of a green fluorescent pseudo-

cells with only one flagellum seems of little weight considering that the term lophotrichous is already partly a misnomer because of the rare occurrence of cells with a real tuft of flagella

If this proposal were accepted it is likely that the existing confusion regarding flagella number in green fluorescent pseudomonads would dissipate and that the flagellar number in *Pseudomonas* taxonomy could be restored to its former status as a useful characteristic

So far these considerations can only be applied to green fluorescent pseudomonads For polarly flagellated organisms other than these the terms mono- and lophotrichous must be retained in their usual vague connotation until it is shown that the more precise definitions also apply to them scattered observations suggest that they do

CONCLUSIONS

1 A flagellar index expressing the percentage of flagellated cells having more than one flagellum per pole is a convenient means of characterizing flagellation in green fluorescent pseudomonads

2 The index value will distribute about 99 per cent of the strains on only two classes a low-index-group having values $\leqq 10$ and a high index group having values $\geqq 25$

3 A division into low-indexed and high-indexed strains is more adequate than a division into mono- and lophotrichous strains as currently defined

4 The established descriptive terms should be conserved, but in order to give them a more precise and adequate meaning monotrichous should be defined as polarly flagellated with an index $\leqq 10$, and lophotrichous as polarly flagellated with an index $\geqq 25$

5 Strains with index values between 11 and 24 constitute less than 1 per cent Examined at 10° C some of these strains disclose their lophotrichous character by acquiring a higher index

6 Use of the proposed definitions for the term mono and lophotrichous will make it possible to re establish flagella number in green fluorescent pseudomonads as a character of taxonomic value

SUMMARY

The number of polar flagella has been studied in stained preparations of 832 motile green fluorescent pseudomonads By using an index to characterize the flagellation two separate classes of strains could be distinguished This distinction is more precise and adequate than a distinction into mono and lophotrichous based on the current vague definitions of these terms Therefore, in order to conserve the already established terms and at the same time obtain the benefit of a distinction according to index value, it is proposed to redefine the meaning of mono and lophotrichous in terms of index value It is anticipated that

at least 5 polar flagella to use the designation lophotrichous and that one of the reasons why he refused to use flagella number in *Pseudomonas* taxonomy was his impression that there were also entities typically having 2, 3 and 4 polar flagella, representing—in his opinion—a type of flagellation intermediate between mono- and lophotrichous. The general experience with pseudomonads in recent works is that a majority of the cells have only 1, 2 or 3 flagella (*Rhodes, Lysenko,* this study). Nevertheless, since *Migula*'s days the term lophotrichous has almost always been defined as having a polar tuft of flagella, although the number necessary to make a tuft is never stated.

Leifson (1951) in his definition of the terms polar mono-, lopho- and multitrichous introduced a new element by claiming that there should be *predominantly* one, respectively more than one, flagellum. (It is confusing that *Leifson* wants to use lophotrichous to describe flagella of a distinctive *shape* and therefore has to introduce the term multitrichous for flagella of the usual shape, but this nomenclatural problem can be disregarded here.) However, *Leifson* did not make it clear what exactly he meant by *predominantly*. Strictly speaking it could mean just more than 50 per cent, but this is hardly what *Leifson* intended it to mean. If *Leifson* by *predominantly* means that at least 85 to 90 per cent of the cells should have either one, respectively more than one, flagellum per cell, it can be seen from the results reported here that his monotrichous group would coincide with the low-index-group of the present study, but the lower limit of his lophotrichous or multitrichous group would—as this study shows—cut right through otherwise homogeneous entities and this would also be the case if the limit was fixed at its theoretically lowest value, *i.e.* 50 per cent. It thus becomes clear that to use predominance of one or the other cell type as part of the definition will result in a line of division (or two lines) which does not fit the observed facts, in other words, the division will be arbitrary.

This analysis of *Leifson*'s definition illuminates the relationship between the terms low-indexed and high-indexed on the one hand and the terms monotrichous and lophotrichous on the other hand. It can be seen that to equate the two sets of descriptive terms would mean that some of the strains which according to present usage are called monotrichous would have to be considered as lophotrichous; on the other hand, it would be obtained that an arbitrary division was replaced by a nonarbitrary one. Therefore, since a division into index groups has the advantage that the groups will be natural ones which can be clearly defined, and since conservation of the terms mono- and lophotrichous has the advantage which accompanies a time honoured terminology, it is proposed to kill two birds with one stone by defining monotrichous as polarly flagellated with an index value ≤ 10 and lophotrichous as polarly flagellated with an index value $\geqq 25$. The semantic objection that some lophotrichous strains will in fact show a predominance of

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STUDIES ON THE CORYNEBACTERIUM ACNES

By

BRONKA BRZIN

Received 5 x 63

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Some characters of these bacteria are particularly controversal The colonies of C acnes on solid media are salmon pink according to *Douglas & Gunter* (1946), rose-red according to *Hauduroy* (1953), greyish-white but may later become pink according to *Wilson & Miles* (1955) and pink according to *Burrows* (1959) *Craddock* (1942) described two types of colonies Type I forms a large heaped up colony, yellowish buff in colour type II a small flat colony

The gelatine liquefaction should be positive (in 10 days) according to *Douglas & Gunter* (1946), but negative according to *Hauduroy* (1953) and *Wilson & Miles* (1955)

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tions

	d Laevulose	d Mannose	Maltose	Lactose	Saccharose	Raffinose	Melizitose	Starch	Dextrin	Glycogen	Inulin	Trehalose	Salicin	Melibiose	Cellobiose
	7	7	0	0	0	0	0	0	0	0	0	21	0	0	0
	6	6	0	0*	0	0	0	0	0	0	0	21	0	0	0
	7	7	0	0	0	0	0	0	0*	0	0	7	0	0	0*
	7	9	7	0*	5	9	7	0	7	0	0	5	0	12	0*
	7	7	6	0*	4	0*	6	0	7	0	0	7	0	0	0*
	8	15	3	0*	6	0*	7	27	+	0	0	7	27	0	0*
	6	6	0	0	0	0	0	0	0	0	0	17	0	0	0
	7	7	0	0	0	0	0	0	0	0	0	17	0	0	0
)	7	10	7	0*	15	0	9	27	7	0	0	18	30	0	0*
0	8	8	7	30	13	0	12	29	6	0	0	19	27	0	0*
3	21	7	0	0*	0	0	0	0	0	0	0	25	0	0	0*
3	21	9	0*	0*	0	0	0	0	0	0	0	15	0	0	0*
0	13	0*	0	0*	0	25	0	0	0	0	0	30	0	0	0*
3	14	0*	5	0	10	10	13	27	23	0	0	5	0	0*	0*
0	7	+	14	0	19	14	5	27	13	0	0	10	0	21	0*

nifies that after 40 days the colour was only slightly yellow

aerobically and anaerobically (with exception of the thioglycollate broth) with or without the presence of CO_2.

The ability of the *C. acnes* strains to produce haemolysis was tested on thin 5 per cent (human sheep horse rabbit) blood agar, incubated anaerobically for 4 to 25 days.

The fermentation and other biochemical properties of the strains were examined in tubes. Two ...
meat-extract p...
medium as pre...
media were po...
oculated with ...
inoculation was ... with Pasteur pipettes. The tubes were incubated aerobically at 37° C.

Twenty nine different carbohydrates (in quantities of 1 per cent) were used in the fermentation tests. In these experiments the Brewer basal medium was used without glucose and resazurin. The reactions were read *every third of fourth day*. The number of days which passed until the blue colour of the indicator brom thymolblue changed to a pronounced yellow is recorded in Table 1. The mark * signifies that at the end of the 40 days' incubation the colour of the indicator was only slightly yellow.

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Strains	Indole	Control	l-Arbutin	Glycerol	i Erythritol	Adonitol	i Dulcitol	d Mannitol	d Sorbitol	Sorbose	i Inositol	l Arabinose	l Xylose	l Rhamnose	[illegible]
1	+	0	0	7	0	0	0	0*	7	0	0	0*	0*	0	
2	+	0	0	7	0	0	0	12	7	0	0	0*	0*	0	
3	+	0	0	6	7	7	0	19	8	0	0	0*	0*	0	
4	—	0	0	3	0	0	0	0*	0	0*	0*	0*	0*	0	
5	—	0	0	4	0	30	0	7	0*	0	0	0*	0*	0	
6	—	0	0	3	7	30	0	0*	0*	0	0	0*	15	0	
7	+	0	0	7	9	30	0	15	7	0	0	0*	0*	0	
8	+	0	0	8	0	0	0	16	15	0	0	0*	0*	0	
9	—	0	0	3	7	0*	0	0	0*	0	0	0*	30	0	
10	—	0	0	2	7	20	0	0	0	0	0	0*	30	0	
11	+	0	0	12	17	25	0	0*	14	0	21	20	28	0	1
12	+	0	0	12	0	0	0	14	0	0	21	0	28	0	1
13	+	0	0	11	23	12	0	14	20	0	0	30	25	0	[illegible]
14	—	0	0	13	25	20	0	0*	0	0	0	25	29	0	[illegible]
15	—	0	0	9	0	20	0	0	0	0	0	28	24	0	[illegible]

The figures in the table indicate number of days until the blue colour changed to a pronounced yellow

needed a thorough study The best method of approach seemed to be the study of authentic cultures

Therefore, the above mentioned and other morphological and biochemical properties were determined on a series of *C acnes* strains Some of the results obtained in this study are reported here

MATERIALS AND METHODS

Strains

Strain No 1 was isolated in pure culture from pus obtained from a clinical case of Acne vulgaris in the Microbiological laboratory of the Medical Faculty in Ljubljana Yougoslavia Most of the other strains were kindly supplied by dr *P Holm* Statens Seruminstitut Copenhagen They were isolated from specimens obtained mostly from patients with Acne vulgaris or Acne conglobata

Cultivation and Tests

The strains were maintained in deep semisolid agar After incubation at 36–37° C for 4 days aerobically the tubes were kept for about 14 days at room temperature before the next subcultivation To test the purity of the cultures the strains were subcultured on plates of thick 10 per cent blood agar incubated aerobically and ... time the various test tubes and plates were inoculated

... was examined morphologically on various solid ... rried out on thick 5 and 10 per cent blood agar ... igar Lewinthal medium nutrient agar and acid ... lucose and glycerol agar with and without car (pH 6-6 5), one and ... bondioxide For the anaerobic incubation the plates were placed in jars (Zeissler s jars) in which after removal of the air by Pfeiffer s oil pump carbondioxide corresponding to 5–10 per cent of the volume of the jar was generated by means of sodium bicarbonate and molar sulphuric acid which had been placed in the jar beforehand

The growth was studied also in fluid media (thioglycollate medium Lewinthal and Wollstein broth 1 per cent glucose broth and nutrient broth) incubated

Fig 1
Small grey and larger yellow colony type in the *Corynebacterium acnes*

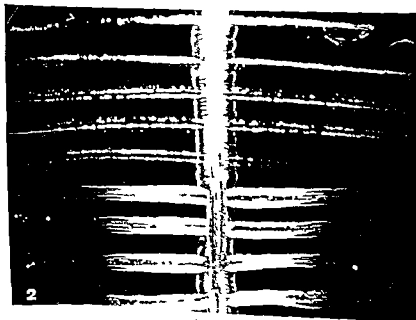


Fig 2
Confluent satellitic growth of the small grey and of the yellow colonies of the *Corynebacterium acnes* around a streak of sarcina under the aerobic conditions

RESULTS

Growth Characteristics

Growth in Fluid Media

The *C. acnes* strains grew very well in thioglycollate medium, in semisolid agar when incubated aerobically and in Wollstein broth, in Lewinthal broth, and in 1 and 2 per cent glucose broth when incubated either anaerobically or in 5–10 per cent CO_2 without oxygen. Growth in the latter media was obtained also under aerobic conditions, particularly with a large inoculum from a young culture. The growth could be obtained also in nutrient broth incubated aerobically with a large inoculum from a culture in thioglycollate medium. After 3 to 5 days, the fluid media were opalescent or cloudy with a fine granular or more coarse greyish-white sediment. Some strains, however, grew more actinomyces-like, *i.e.*, as granules with a nearly clear supernatant.

Growth on Solid Media

The growth of various strains on solid media varied even more. Incubated anaerobically most strains grew in small and medium sized (*1–2 mm in diameter*) *grey or greyish-white colonies*, the colour of other colonies being more yellowish. The strains 6, 9, and 10 grew in distinctly yellow colonies. The colonies of strain 4 were salmon pink. The pink colour was more pronounced on Löffler's medium, on glucose and on glycerol agar. On glucose and on glycerol agar a propionic acid smell developed after 5 days of anaerobic incubation. This smell became more pronounced in the following days of aerobic incubation.

The majority of the strains could be grown on solid media aerobically if the inoculum was large and the plates of blood agar (better 10 than 5 per cent) were thick enough to permit a sufficiently long aerobic incubation without dessication of the plates. Only few strains grew aerobically from a small inoculum. Aerobic cultivations were most successful on plates of slightly acid glycerol agar. However, the colonies which developed under aerobic conditions were fewer than after anaerobic incubation of the same inoculum. On the other hand the colonies often attained larger dimensions (0.6 or 0.8 cm in diameter) after prolonged (2 weeks) aerobic incubation than after the ordinary (5 days) or even 10 days anaerobic incubation.

In one strain, a confluent aerobic growth was obtained around a colony of sarcina. This strain of *C. acnes* dissociated into two types of colonies which could be separated into pure culture: large dark yellow and small greyish white colonies (Fig. 1). Both types of colonies showed the satellitic phenomenon of aerobic growth around a streak of sarcina (Fig. 2). In the cultures of the same strain a lysis by phages was observed for a long period of repeated subcultivations in semisolid and on solid media. The action of these phages was evident not only in the

Fig 1
Small grey and larger yellow colony type in the *Corynebacterium acnes*

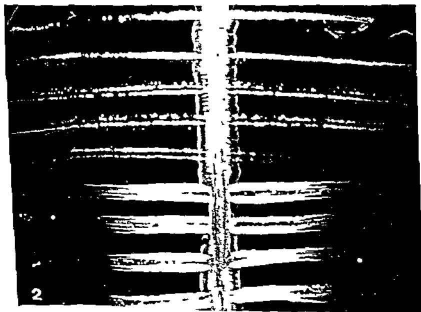


Fig 2
Confluent satellitic growth of the small grey and of the yellow colonies of the *Corynebacterium acnes* around a streak of sarcina under the aerobic conditions

ordinary type of lysis in plaques with a secondary central colony (Fig 3) but also in another phenomenon Some sectors of the yellow colony became progressively greyish transparent and flatter as if undermined (Fig 4) The convex and the yellow parts of the colonies retained their shape and colour for a different period of time but finally they followed the changed parts in colour and outline The described changes could be followed also under aerobic incubation The phage particles could be demonstrated in the filtrate of such "denuclated" colonies electronmicroscopically The shapes seen on the electronmicrography did not differ from those of other phages

Haemolysis

All strains, except No 4, which was nonhaemolytic during 3 weeks of anaerobic incubation and showed slight traces of haemolysis only in the fourth week of incubation, showed beta haemolysis in 5 or 10 days if incubated anaerobically The haemolysis was more pronounced and occurred faster in the strongly proteolytic strains 6, 9, and 10

Biochemical Reactions

The results of the fermentations of the carbohydrates and of the indole production are given in the table of biochemical reactions The fermentations of the carbohydrates in the semisolid agar and in the thioglycollate medium corresponded fairly well Only minor differences were observed in some of the tests and in those cases the mean values was calculated and recorded in Table 1

As shown in the table, all strains produced acid in glycerol, d-glucose, d galactose, d-laevulose, d mannose, and in trehalose None of the strains tested produced acid in l-arbutin, dulcitol, sorbose, l-rhamnose glycogen, and inulin

In addition to the above mentioned carbohydrates which are fermented by all the strains of *Corynebacterium acnes* examined, some other carbohydrates (maltose, saccharose, melizitose, and dextrin) are fermented fairly quickly by all the strains which do not produce indole

Starch and salicin are slowly fermented only by the strains 6, 9, and 10 which have a strong proteolytic and haemolytic action

The indole reaction was positive in 2 or 3 days with the indologenic strains If negative at this time it remained so in the same and in other media on the following days The indole reaction marked as positive in the table indicates a positive reaction in semisolid agar, incubated aerobically, in Lewinthal and in Wollstein broth incubated anaerobically and aerobically, in tryptose and in nutrient broth incubated an aerobically and also aerobically provided that the strain could be grown in these media under aerobic condition In all these media the indole test became positive after 2 or 3 days of growth in the semisolid agar after 3 days of incubation Only in the fluid and semifluid thioglycollate

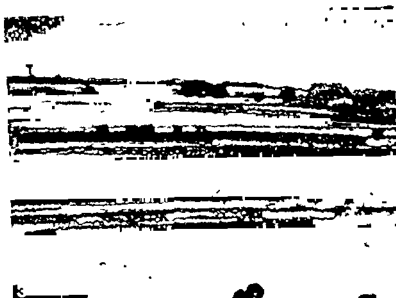


Fig 3
Lysis of the colonies of the *Corynebacterium acnes* in the form of plaques with the central secondary colonies

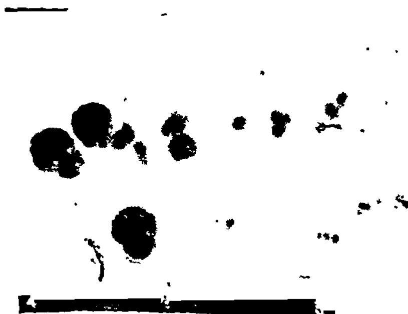


Fig 4
The second phenomenon of the phage action in a strain of *Corynebacterium acnes* some sectors of the yellow colonies became progressively flater and greyish transparent

medium, with glucose, the indole test was always negative also on prolonged incubation (to 30 days) and even with a good growth of strains which were indologenic in other media Addition of tryptose did not induce indole production in this medium with glucose However if glucose was *omitted* the strains were found to produce indole also in the thioglycollate media

The results of the nitrite production tests correspond in general to the results of the indole tests However, the results were not so clearcut as in the indole production tests They also varied somewhat when repeated with the same strains in the same media

Even greater variations were obtained in the other tests For this reason these results are omitted here It can only be said that proteins (coagulated serum, egg albumin and gelatine) are lysed at a different rate by the various strains of *C acnes* examined

DISCUSSION

The phages in the anaerobic Corynebacteria were found for the first time in 1956 by *Mandin* (*Prevot* 1960) in a culture of *Corynebacterium parvum* The lysis was observed and studied only in fluid and in deep solid media but not on the surface of solid media The electronmicrophotography showed a morphological pattern similar to that found in the present study

The present results of the fermentative reactions correspond mainly to those described elsewhere (*Hauduroy* 1953) glucose, galactose, and glycerol are always fermented, saccharose, lactose, mannitol, and dextrin may be negative or positive Maltose was found to be fermented by some strains and not by others, in contrast to the always positive maltose fermentation found by other workers (*Hauduroy* 1953)

In the present study maltose was fermented only by the strains which *did not* produce indole Beside maltose, some other carbohydrates (saccharose, melizitose, and dextrin) were always fermented by the strains which did not produce indole and did not reduce nitrates to nitrites, while no fermentation was obtained with the indologenic strains Starch and salicin were fermented by most of the saccharolysers Therefore, the following conclusions seem to be permissible The strains of the *C acnes* which do not produce indole ferment a larger number of the carbohydrates than the indologenic strains On the basis of the fermentative reactions and of the indole production, *i e*, the biochemical reactions which were found to be most consistent in the *C acnes* strains, this species could be divided into two biochemical types, the saccharolytic and the indologenic type

About one-half of the strains examined produced indole This corresponds to the findings of *Douglas & Gunter* (1946) Correspondence of the positive indole and nitrite tests in the strains is in accordance with the data in Prevot's table of the biochemical reactions of the anaerobic

Corynebacteria (*Prevot* 1960) but not with the findings of *Douglas & Gunter* (1946)

None of the examined anaerobic Corynebacteria could be systematized according to *Prevot*'s classification of the anaerobic Corynebacteria (1960) The anaerogenic, strongly proteolytic strains (No 6, 9 and 10) may correspond to the Corynebacterium liquefaciens but they do not produce indole and do not reduce nitrates They can be induced to grow aerobically They are not strict anaerobes as claimed (*Prevot* 1948 *Prevot & Tardieux* 1953, and *Prevot & Courdurier* 1949) for the anaerobic Corynebacteria except the *Corynebacterium acnes* This claim, however is no more absolute in the recent research work (*Prevot* 1960)

Seeliger seems not to accept the classification of *Prevot* He wrote (1953) that cultures recognised as *C liquefaciens* were identical with cultures of *C acnes*, isolated by him and with strains of the same organisms isolated and identified by *Lentze* (1950)

It should also be stated that thioglycollate medium without glucose and semisolid agar are satisfactory for studies of biochemical reactions in the Corynebacterium acnes

SUMMARY

Various types of macroscopic growth of *Corynebacterium acnes* strains on solid and in fluid media are described

Two types of phage action were observed, first, plaques with the secondary central colonies, and secondly, the denivelation and change in the colour of the colonies It was assumed that the colony type dissociated into two under the influence of the phages

A strain of sarcina was found to enhance the aerobic growth of both types of the colonies The oxygen tolerance studies indicated that *Corynebacterium acnes* strains may grow aerobically after a large inoculum The readiness of the individual strains to grow aerobically depends on the strain and on the medium used

On the basis of fermentative reactions and of indole production i e, the biochemical reactions which were found to be most consistent in the *Corynebacterium acnes* strains the latter could be divided into two biochemical types the saccharolytic and the indologenic type

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